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STUDIES ON BOVINE MASTITIS.

VII.—THE SEROLOGICAL CHARACTERS OF MASTITIS STREPTOCOCCI.

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A STUDY of the serological characters of bacteria is of value because it reveals the basic pattern on which a given species is built, and enables one to demonstrate the true relationship between morphologically similar organisms, isolated from various sources in the same or in different individuals. Serological methods are the most sensitive because they have revealed definite and constant differences between

strains which are by all other tests similar ; and, although repeated propagation under artificial conditions may give rise to various forms from one strain, there seems no reason to doubt that the antigenic characters of recently isolated strains can be accepted as useful criteria. Moreover, they are criteria of considerable biological significance and are of the utmost importance in the field of immunisation.

The purpose of the present paper is to describe the serological characters of streptococci isolated from the secretion of the bovine udder affected with mastitis, and to correlate these with the behaviour of the strains towards blood, and in various biochemical tests. In Part I, the methods will be described ; in Part II, the results of the examination of 160 strains will be given.

In previous reports of this series (1929, 1931, 1932) two groups of hæmolytic streptococci and three groups of non-hæmolytic streptococci from mastitis have been described. The latter have been called Groups I, II and II respectively. It has been shown that the larger hæmolytic group and the non-hæmolytic Group I, apart from their effect upon blood, correspond closely in general characters, and the work now reported shows that the two are similar serologically. It will also be shown that each of the groups distinguished on biochemical grounds contains more than one serological type, but that the several types within each group are serologically distinct from those of other groups.

Consideration of the smaller hæmolytic group will be deferred until a later paper.

The strains were obtained by cultivation in blood agar plates, isolated colonies being transferred to 1 per cent. serum broth, from which, after examination for purity, subcultures were made in a cooked meat medium for storage in the cold. This was renewed at three to six-monthly intervals and from it were sown all media required for serological or other tests.

PART I.

Choice of Methods.

The methods employed in serological examination were (*a*) precipitation at 37° or 55° C., extracts prepared in hot M/20 HCl. being used ; (*b*) a slide agglutination technique at room temperature, and the corresponding absorption tests. These were chosen after a survey of the numerous reports dealing with the possibilities of a serological classification of streptococci. A detailed discussion of the results obtained with scarlatinal and other streptococci of human origin would be out of place here, but reference may be made to those which had a major influence on the choice of methods to be used in this work.

Kinsella and Swift (1917, 1918), using complement fixation with antiformin extracts from 56 hæmolytic and non-hæmolytic strains of varied biochemical character, concluded that the hæmolytic strains were all nearly similar, whereas non-hæmolytic strains, although showing a considerable amount of cross fixation, were much more heterogeneous. They found a certain degree of likeness between one section of these and the hæmolytic strains.

Hitchcock (1924, 1925), using somewhat similar methods, and including a precipitin test for the non-hæmolytic strains, came to rather similar conclusions.

Dochez, Avery and Lancefield (1919), using agglutination, found that out of 125 strains of scarlatinal streptococci 85 could be placed in four main groups. Mouse protection tests carried out with the virulent strains agreed in showing a similar grouping. From their comments and those of Lancefield at a later date it is clear, however, that considerable difficulty was found in obtaining suitable suspensions for agglutination tests.

Stevens and Dochez (1924, 1926), using methods based on those last mentioned, but with the addition of tests for absorption of agglutinins, found a less sharp division into types and concluded that the agglutinogens were of a complex nature with characteristic variation according to source.

Griffith (1926, 1927) showed that by agglutinin-absorption scarlatinal streptococci could be separated into well marked types and that by means of a slide agglutination test carried out under a dissecting microscope useful preliminary orientation could be made, even with suspensions which were too granular for the ordinary tube agglutination test.

Smith (1926, 1927) obtained clear differentiation by the standard agglutination test, using streptococci grown in salt-free buffered broth.

Lancefield (1928) re-examined a number of the strains originally classified by Dochez, Avery and Lancefield, and did much to clarify the conflicting nature of the results obtained by various methods, by showing that sera prepared with whole streptococci contain at least three and probably four distinct antibodies. One is induced by the nucleoprotein which she found was a common factor in all streptococci and related to that found in staphylococci and pneumococci. A second is elicited by the carbohydrate fraction and is species-specific. The third is brought forth by a fraction of protein nature and is one of importance in classifying by means of precipitin reactions with extracts prepared in hot M/20 HCl. The results which she obtained by this method corresponded closely with those obtained earlier by Dochez, Avery and Lancefield, using agglutination and mouse protection tests.

Lancefield's method was chosen at first because it got over the difficulty of spontaneous agglutination or granularity which has hampered the use of the agglutination test, and because it seemed likely that it would reveal different types amongst the mastitis streptococci, if such existed. About 50 strains were tested by this method before any other was tried. By this time it had become clear that, owing to the large amount of serum and media required and the difficulty of obtaining suitable sera, this method would, for practical reasons, be inapplicable to a large number of strains. Tentative experiments were, therefore, made with a slide agglutination technique. Apart from economy in time and material, this method is peculiarly suited to work with streptococci because it can be applied to the great majority of freshly isolated strains without recourse to

any of the methods which have been developed in order to obtain suspension that are stable for the one or two-hour periods at 55° C. required by the standard tube test.

In order to overcome the difficulty of spontaneous agglutination many methods have been used, most of which necessitate frequent or continued subcultivation, a procedure which, as has been shown by F. W. Andrewes (1928) and by Todd and Lancefield (1928), may result in a decrease of specificity. Cultivation at room temperature is not practicable and in salt-free broth a number of strains which are quite unsuitable for tube tests at high temperature are still encountered. Even when the agglutinin-absorption test is used it may be difficult to obtain stable suspensions of the type strains with any constancy, and in any case agglutinin-absorption is time-consuming and, for the examination of large numbers of strains, needs to be supplemented by some method by which entirely dissimilar strains can be eliminated. With this object in view a macroscopic slide agglutination test at room temperature was adopted and it was found that tests for absorption of agglutinins could also well be made by this method. The slide method is not one of absolute precision, but it is rapid and has provided strikingly reproducible results and, in subsequent work not reported in this paper, has proved of great value in deciding whether further tests are necessary.

Both the precipitation and agglutination tests have been applied to nearly all the streptococci considered in this report. The results were in close agreement ; though in at least one group of streptococci differences were apparent, due probably to the fact that more than one antigen may be concerned in agglutination and the corresponding absorption.

Antigens and Sera.

Extracts.

The deposit from an 18 to 24-hour culture in 1·0 per cent. glucose broth was suspended in sufficient M/20 HCl. in saline to give an opacity equal to that of a kaolin suspension of which 10 c.c. gave 0·2 c.c. deposit. The suspension was immersed in boiling water for 15 minutes and frequently shaken. It was then cooled, neutralised in the presence of phenol-red, and centrifuged. To the clear supernatant was added one-tenth of its volume of 1·0 per cent. chinosol as a preservative. The volume of extract obtained from each 100 c.c. of glucose broth varied greatly with the strain and also with the batch of medium, but averaged about 5 c.c.

Comparative experiments with a few strains showed that extracts of equal potency were obtained from glucose and from plain broth, and from suspensions extracted for 15 and 20 minutes respectively. Extraction for ten minutes resulted in a product of slightly less value. Within the range *p*H 6·6 to 8·0 the reaction had no immediate effect, but after storage at *p*H 8·0 for one month deterioration was detectable, and after six months it was marked. This is in accordance with

other observations that an extract which becomes alkaline either from contact with glassware of poor quality or from contamination, gradually deteriorates. The loss cannot be restored by neutralisation.

An extract stored under sterile conditions will retain its activity practically undiminished for at least four months, and frequently after storage in the cold for 12 to 18 months the greater part remains. However, as extracts have to be used frequently for control purposes, it is convenient to add some antiseptic. Comparative experiments in which one-tenth of their volume of sterile saline, 1 per cent. chinosol, or 5 per cent. phenol, was added to extracts of representatives of most of the types, showed that neither chinosol nor phenol caused any decrease in potency within nine months. Chinosol was generally used.

Agglutination Suspensions.

At first these were always grown in plain salt or phosphate broth, but in the later work broth containing horse serum or glucose was also used. Serum not only increased the yield but somewhat repressed the tendency of certain strains to clump spontaneously, 1·0 per cent. being generally sufficient, though with certain unstable strains it was of advantage to add 5 or even 10 per cent. Such large amounts were only used in particular cases, because there was some evidence that they interfered with specific agglutination of a strain which was very stable. 1·0 per cent. glucose broth was usually satisfactory even when not buffered. Neutralisation was effected by means of N/1 and N/20 NaOH in the presence of phenol red.

Media were not incubated longer than necessary to get sufficient growth. The organisms were obtained by sedimentation or centrifuging and suspended in sufficient 0·1 per cent. chinosol in saline to give a density ten times that of a Brown's standard opacity tube No. 8, *i.e.*, approximately 30,000 million streptococci per c.c. Suspensions thus made deteriorated slowly and were agglutinated to practically the same titre for some months; their type could often be recognised even after one or two years. Certain suspensions, however, became more apt to clump spontaneously.

Immune Sera.

So far sera of thirteen types have been prepared.* In all, 56 sera were prepared in rabbits and 24 in goats; of these in each case about half proved useful for precipitation and rather more for agglutination, which does not necessitate a serum of such high antibody content. Probably the majority could have been used for agglutination, but were not, because better sera were available or because in the earlier work the precipitin method was in use chiefly or solely.

* Four of these types are not detailed in this report. They do not belong to any of the groups described, with strains of which their sera gave uniformly negative results.

Streptococci for inoculation were grown in plain broth or, when large amounts were required, 1·0 per cent. glucose broth. They were used in the living state or after killing by heating at 58° C. for one hour, or by means of 4 to 5 parts per 1,000 of formalin. Formalised cultures had, however, no obvious advantage over heat-killed. There has been no evidence that killing with formalin or cultivation in glucose broth interferes with specificity. The intravenous route was generally used. Injections were made once weekly or on two or three successive days of each week, the dose being gradually increased from 0·1 c.c. to 10 c.c. for rabbits, and from 1 c.c. to 100 c.c. for goats. Except in the case of the smaller doses the cultures were centrifuged and the deposits re-suspended in saline.

In goats, strains of Group II were always killed because living organisms invariably produced joint lesions even though the animal had received a course of dead vaccine previously. In rabbits inoculated with similar strains no such lesions occurred. Fortunately, good sera of this kind were easily obtained with dead organisms.

Sera were stored in the cold, usually after the addition of 0·01 per cent. of chinosol.

Technique of Tests.

Precipitation.

In connection with precipitin tests in general it has long been recognised that the relative proportions of antigen and antibody are of importance. For this reason in the earlier work both serum and extract were varied. It soon became clear, however, that, whilst 0·2 c.c. of extract gave definite results with a suitable amount of immune serum, 0·4 or 0·5 c.c. was a more satisfactory volume because it gave ample precipitate with a suitable amount of homologous serum and was moreover not likely to cause inhibition in the presence of any reasonable amount of antibody. It is, of course, possible to so reduce the antibody that it will react with 0·1 c.c. of the homologous extract and not with 0·4 or 0·5 c.c. In later work, therefore, as soon as a particular serum was harvested, a preliminary titration was made with 0·5 c.c. of the homologous extract to find approximately the lowest volume of serum that could be used, and this volume and one twice as great were tested with varied amounts of extract. According to the results, the serum was used in one of these doses or tested in a yet larger volume.

Examples of titration of serum and extract from S57, S32, S84 and N18, representing a few of the more important types, are given in Table I.

In the case of each serum and the corresponding extract the tables show a zone of delayed flocculation at the left hand bottom corner due to excess of serum and another, more pronounced, at the right hand top corner due to excess of antigen. The final amounts of precipitate show no diminution due to excess of serum. On the other hand excess of antigen has decreased or completely prevented flocculation, particularly when the amount of antibody present was low.

It is evident that 0·25 c.c. of serum S57 is a suitable amount to use ; it will detect amounts of antigen of the same type varying from one quarter to five times that in the homologous extract. It would not be safe to use 0·1 c.c. of this serum because 1·0 c.c. of extract caused complete inhibition of flocculation ; and consequently 0·5 c.c. of an extract of an unknown strain, which though made from a suspension of the same density might have a greater antigenic content, would be likely to produce little or no precipitate and remain unrecognised even if the same type. Similarly, 0·1 c.c. of serum S32 is a safe amount, and 0·25 c.c. of serum S84 can be used with care ; on the other hand, serum N18 would be unreliable, even in a volume of 0·5 c.c.

Although it was necessary to carry out these titrations in order to guard against inhibition by extracts of unusual antigenic potency, the results have shown that, with a few exceptions, extracts prepared in the manner described, from different growths of the same strains or from strains of the same type, gave a notably constant amount of precipitate with any given serum. In cases where an unusually small amount of precipitate was obtained with one type of serum only, or in which some precipitation occurred with sera of more than one type, further tests were set up with larger and smaller volumes of extract.

Table I also shows that in the titration of the serum and extract from S32 a secondary zone of precipitation is present. This appeared late and was not of a flocculent character ; its nature is not clear.

Agglutination.

The actual tests were carried out at room temperature on ordinary slides on which were outlined six cells, 1-in. × 0·3-in. by means of a stencil and grease pencil. All amounts were measured by drops from pipettes which delivered 30 drops per c.c. Sera, absorbed or unabsorbed, were distributed first and drops of suspension placed close to them. The surface of the slide was moistened by gently breathing on it, the slide gently rocked in various directions once a second and the results watched against a dark background under suitable lighting conditions. Readings were made from the time of the first appearance of agglutination and at intervals up to 60 seconds when the final reading was taken. Actually with suspensions of many types it is possible to make readings up to five minutes or longer ; but, in view of the fact that many suspensions spontaneously clumped sooner than this and that when testing suspension with homologous sera little or no increase of agglutination occurs after one minute, readings were not usually prolonged beyond that period.

New strains were tested first with single dilutions of sera made specific for each type, the dilutions being chosen so that they clumped their respective homologous suspensions within a somewhat similar time. Usually the results were clear, agglutination being absent

TABLE I

Optimal Proportions.

Variable serum and extract, mixed and read at intervals during incubation at 53° C. and after 1½ hours at this temperature. Final readings after a further 17 to 18 hours at about 5° C.

Serum and Extract S57.

Time of Readings.		5 *mins.*			10 *mins.*			15 *mins.*			40 *mins.*			*Final.*		
Volume of serum (c.c.) ...		0·5	0·25	0·1	0·5	0·25	0·1	0·5	0·25	0·1	0·5	0·25	0·1	0·5	0·25	0·1
Volume	2·0 ...	—	—	—	+	—	—	+	—	—	+	—	—	4	<1½	—
of	1·0 ...	±	—	—	+	—	—	+	—	—	+	+	—	3	1½	—
Extract	0·4 ...	+	±	—	+	+	—	+	+	—	+	+	±	2	1½	1
in c.c.	0·1 ...	—	—	+	—	—	+	—	+	+	+	+	+	<1	<1	½

Serum and Extract S32.

Time of Readings.		12 *mins.*			18 *mins.*			25 *mins.*			1½ *hours.*			*Final.*		
Volume of serum (c.c.) ...		0·25	0·1	0·05	0·25	0·1	0·05	0·25	0·1	0·05	0·25	0·1	0·05	0·25	0·1	0·05
Volume	2·0 ...	+	—	—	+	—	—	+	—	—	+	turb.		6	? 2 turb.	2 ? type.
of	1·0 ...	—	—	—	+	—	—	+	—	—	+	+	—	4	3	tr.
Extract	0·4 ...	—	+	—	—	+	+	±	+	+	+	+	+	3	2	1
	0·1 ...	—	±	—	—	+	±	—	+	+	±	+	+	1	½	½

SERUM AND EXTRACT S84.

Time of Readings.			40 *mins.*			1½ *hours.*			*Final.*		
Volume of serum (c.c.)		...	0·5	0·25	0·1	0·5	0·25	0·1	0·5	0·25	0·1
Volume of Extract	2·0	...	—	—	—	+	—	—	4	—	—
	1·0	...	—	—	—	+	+	—	4	2	—
	0·5	...	+	—	—	+	+	+	3	2½	1
	0·1	...	—	—	—	+	+	+	1	1	1

SERUM AND EXTRACT N18.

Time of Readings.			1½ *hours.*			*Final.*		
Volume of serum (c.c.)		...	0·5	0·25	0·1	0·5	0·25	0·1
Volume of Extract	1·0	...	—	—	—	—	—	—
	0·4	...	±	—	—	1	tr.	—
	0·1	...	±	tr.	—	1	½	tr.

or occurring with one type only. Positive results were confirmed by tests at higher dilutions and by absorption test. No zone phenomenon was seen.

The titres obtained by the method described varied from 1/80 to 1/1,280 according to the serum.

In the case of a few type strains which gave stable suspensions the titres obtained by the slide method were compared with those obtainable in tubes. The suspensions used for the tube test contained 3,000 million bacteria per c.c. and were distributed in amounts of 18 drops into small tubes. Two drops of one of the serum dilutions used for slide tests were added to each tube, which was immediately shaken. The tubes were placed in a water bath at 55° C. and read at intervals up to two hours.

The results showed that when the same proportions of antigen and antibody were present the results obtained by the two methods were nearly similar. The titre, *i.e.*, the actual dilution of serum which caused similar agglutination, was nearly ten times as great in tubes using the more dilute suspension as on slides. In the case of the sera tested it was 1/400 to 1/12,800.

Preparation of Type-specific Sera.

In order to make a given serum specific for the type used in its production it was treated with a strain of each other type with which it was found to give cross-reactions. These reactions were much more in evidence in agglutination tests than in precipitation tests and necessitated a proportionately greater amount of preliminary absorption.

When required for agglutination the serum was diluted ten times and mixed with a sufficient amount of killed bacterial bodies from an 18 to 24-hour plain or glucose broth culture of each strain. In normal circumstances, when the specific antigens of the serum strain and of the absorbent were entirely different, 0·2 c.c. of deposit was used for each c.c. of serum. The absorption mixtures were incubated at 37° C., for one hour or at 55° C. for a shorter period and centrifuged immediately or after standing overnight in the cold. Little or no reduction of specific titre resulted. Occasionally it was necessary to repeat the absorption with one or more types. Sera for use in precipitation tests were similarly treated, except that the serum for absorption was undiluted or diluted not more than three times. Serum diluted ten, or often even three, times would be unsuitable for use in precipitin tests.

It should be added here that certain strains were of particular value for preliminary absorption in that they removed non-specific antibodies not only for their own type but for other types of the same group. This is probably because their make-up is a mixed one, including not only a major amount of antigens of one type, but also a minor amount of other antigens.

Absorption of Homologous Antibodies.

In order to confirm the results of direct tests, a number of strains of each group, or in some groups all strains, were used to absorb one or more sera of the type to which they presumably belonged.

Precipitation.—The serum was diluted two to three times, or in the case of a poor serum used undiluted. A suitable volume was absorbed at 37° C. for one hour with a volume of deposit equal to, or only a little more than, that amount of deposit from the serum strain which just prevented the formation of any precipitate when the absorbed serum was mixed and incubated with 0·5 c.c. of the corresponding extract. Tests with 0·2 or 0·1 c.c. of extract indicated that sometimes sufficient antibody was left to produce a slight precipitate with one of these amounts of antigen, but for the purpose of routine absorption this was ignored. In many cases two or three amounts of absorbing deposit were used in parallel with similar amounts of the serum strain. In certain cases, where only one amount of absorbent was used, a second absorption was required. In general it was found that a little less of the serum strain was required than of other strains of the same type.

Agglutination.—From the results of direct agglutination a suitable dilution of serum was chosen and a given volume of this, usually six or twelve drops, was mixed with an equal volume of agglutination suspension or with smaller amounts made up to the same volume with the usual diluent, 0·1 per cent. chinosol saline. When it was desired to use very large doses for absorption the organisms were deposited, and re-suspended in a volume equal to that of the serum dilution. Absorption mixtures were incubated for ten minutes at 55° C. or longer periods at lower temperatures, with occasional shaking. The absorption was controlled with one or two homologous and heterologous strains. A given volume of serum dilution was absorbed nearly equally by strains of the same type. Twelve times the minimal absorptive dose as determined with homologous strains did not cause a detectable amount of absorption if the strain was of another group. In the case of certain types within the same group, however, some cross-absorption of specific antibodies did occur when these large doses were used. Absorption, if any, and its approximate degree was nearly always obvious from a single slide test without further dilution of the absorbed serum. In cases of doubt, and as a check in other cases, absorbed sera were tested to their end point, but usually no more information was obtained than by the comparative speed of agglutination, if any, at the dilution actually used for absorption. This dilution was so chosen that complete agglutination would occur within about 15 to 30 seconds when no absorption occurred.

Discussion.

The precipitation method described, which depends on the extraction of a fraction of protein nature soluble in hot M/20 HCl.,

has proved applicable to all types of mastitis streptococci except those of Group III.

The precipitates which may appear are, however, of three kinds. One, produced by the nucleoprotein antigen and antibody, is of a non-specific nature, is often large and definitely flocculent, and usually appears later than other precipitates. A second precipitate, produced by the interaction of the carbohydrate fraction and its corresponding antibody, appears first as a slight opacity, but is usually deposited as a small semi-transparent disc which adheres to the bottom of the tube and can be detached whole by twisting the tube vigorously. The third, that occasioned by contact of the type-specific protein factor with its corresponding antibody, appears quickly compared with the nucleoprotein precipitate, though at different times, according to the character of the serum and the strain that produced it, it flocculates rapidly, is usually distinctly white and is easily re-suspended as larger or smaller particles which coalesce again more or less quickly according to the temperature.

The carbohydrate precipitate has not been a common source of trouble in this work. It only appeared when large volumes of certain sera, poor in the specific element, were being used. Its characteristic nature, moreover, makes confusion unlikely. I have not experienced the same amount of trouble due to nucleoprotein precipitates as Lancefield appears to have done. This may have been due to the higher temperature of incubation used in this work, since that certainly accelerates the specific precipitate, or it may have been due to the different nature of the strains. Since, however, at certain times confusing precipitates did appear when sera were tested with strains of known type, tests of unknown strains were carried out with sera made specific by preliminary absorption; or, if tests were first made with unabsorbed sera, positive results were confirmed with absorbed sera or by specific absorption of the appropriate serum.

It is natural that attempts should have been made to produce sera by immunisation with the type-specific protein only. Unfortunately, it has been found impossible to produce antibody response with any fraction of the streptococcus other than the nucleoprotein.

It seems probable that the streptococci of Group III, which are distinguished by the fact that they ferment mannite, are actually deficient in the specific protein fraction. This view is based on the fact that, whereas all sera of moderate agglutinating titre produced by immunisation with other strains have always given typical precipitates with the corresponding extracts, sera of similar agglutinin titre produced by means of Group III strains have never given the precipitate characteristic of the type-specific protein, but, if any precipitate at all, one which was of the nature recognised as produced by the carbohydrate factor.

The slide agglutination method has proved applicable to nearly all strains, both as a test of direct agglutination and of absorption.

The effect on a given suspension of various dilutions of one serum, of equivalent dilutions of different sera, or of the same dilution of one serum in the unabsorbed or absorbed state, can be compared side by side and read at the most suitable times. This is of great advantage when dealing with suspensions of such diverse stability as those of streptococci, and enables one to decide the type of suspensions which are quite unsuitable for the tube test. With sera rendered specific by preliminary absorption the results are generally clear, agglutination occurring with one type of serum only.* Tests for absorption of agglutinins are quickly carried out with minimal amounts of suspension and serum and the results are easily read, complete or partial absorption if present usually being obvious at the dilution actually used for absorption, and confirmed by further tests with dilutions of the absorbed serum. Replicate absorptions, carried out at once or at weekly or monthly intervals, have given similar results.

Suspensions prepared from the same strain at different times are not always equally agglutinable, and in the case of certain strains the difference is striking. There is some reason to believe that the rate of growth and duration of incubation are important factors in this connection, and Spicer (1931) has reported that very young cultures, though less liable to spontaneous agglutination, are much more agglutinable by specific antibodies. I have, however, not noticed any regular relation between rate of growth or duration of incubation and agglutinability. In this connection it may be recalled that Mueller and Klise (1932), while finding that 48 hours was usually the most suitable period of incubation, observed in the same streptococcus culture a remarkable sequence of spontaneous agglutination and complete inagglutinability followed by perfect specific agglutination. These workers used glucose broth cultures and apparently it was not necessary to neutralise them. The experience in this work has been that cultures in glucose broth are frequently of very low agglutinability until they are neutralised, when they ususally form suspensions as good as those grown in any other medium. Certain strains are, however, very unstable before neutralisation. It has not been possible to correlate these differences in stability with the acidity limiting growth. Mueller and Klise also report that the addition of normal serum may inhibit spontaneous agglutination. This is certainly the case, and many suspensions are stable in serum dilutions up to 1 : 50 or 1 : 100 whilst agglutinating spontaneously at higher dilutions or in saline ; but there are many suspensions which are unstable under all conditions.

Inagglutinable suspensions or suspensions of notably low agglutinability have, however, only been encountered in Group II. It is

* In the case of certain strains it was necessary to prepare more than one suspension before a stable one was obtained. Unstable suspensions usually give similar results with all sera other than the homologous serum; but occasionally, probably owing to the fact that they are hypersensitive, the degree of agglutination with various heterologous sera is different.

hoped to investigate this phenomenon at a later date, but it may be said here that even the rare totally inagglutinable suspensions absorb perfectly, and that their extracts precipitate normally with homologous serum. This suggests that the phenomenon is not due to dissociation but to some interference with the second or non-specific phase of agglutination.

It will be noted that the strains are referred to as groups, each of which contains various types. The term group was already in use with regard to biochemical characters; its retention is not inappropriate because there is a certain parallelism between biochemical and serological groupings. This, however, does not necessarily imply serological likeness between types within the same group. In many cases some degree of likeness actually exists, but in others the antigenic make-up is entirely different.

In carrying out comparative agglutination tests with absorbed and unabsorbed sera it soon became obvious that preliminary absorption in order to make the sera type specific was a necessity, because, although with most sera distinctions could be drawn between the various suspensions when high dilutions of serum were used, there was considerable cross agglutination at the lower dilutions. These cross-reactions were, however, much more marked between types within the same group than between types of different groups; in fact, it was unusual to find any cross-reaction between types of different groups and rare to meet with a reaction at dilutions above 1/5 or 1/10. In conformity with these observations, absorption of a serum with a member of another group never resulted in a notable lowering of titre, even when the absorbent was grossly excessive. On the other hand, absorption with a member of another type within the same group did, in many instances, result in a distinct lowering of titre, particularly if the absorbing organisms were more than three times the minimal absorption dose as estimated from tests with the serum strain. In no case, however, did complete absorption occur, although the amounts used were in many cases at least ten times the minimal absorptive dose; moreover, in most cases the amount of absorption did not increase with the absorbing dose beyond a certain point. It seems, therefore, that strains of different groups are antigenically entirely different, whereas strains of different types within the same group may possess, in addition to the dominant antigen responsible for their type characters, a certain amount of other antigens in common with one another.

Sera which gave cross-reactions in agglutination tests were frequently specific as regards precipitation tests, and a large number of extracts were tested with unabsorbed sera with perfectly clear-cut results, a precipitate being obtained with sera of one type only and confirmed by specific absorption. At times, however, certain unabsorbed sera, notably those of Group II, did give marked precipitates with strains which were definitely of another type; these non-specific reactions were not confined to members of the same

group and the character of the precipitates suggested that they were due to nucleoprotein. It may be that the factor involved in these non-specific precipitates is the same as that concerned with the non-specific agglutination reactions, and that the difference is due solely to the relatively smaller amount of antibody required to coat the larger particles of an agglutination suspension. However, in view of the results with the streptococci of Group III, with which typical precipitates were never obtained, and of those with Group II, it seems more likely that this distinction is due rather to a difference in the fraction of the streptococcus producing the antibody concerned. In Group II the results of precipitation and agglutination tests were not fully parallel, and repeated absorption of the only Group II serum tried with its corresponding extract did not affect the agglutinating titre. This is in contrast with the findings in Group I, and work on this point is being continued.

In picking colonies from plates seeded with mastitis secretion it is unusual to find organisms of more than one kind. The original flora seem actually to disappear or at any rate are so outnumbered by the pathogens that they are generally absent on plates containing 100 to 500 colonies. From such plates it has been the usual practice to pick two or three colonies and, if these have the same general and biochemical characters, to accept them as representatives and to store one culture only. In the earlier part of this work, therefore, only one strain from each sample of secretion was available. In subsequent work it was felt advisable to examine a number of colonies from the same plate in order to be assured that one or two of them could be accepted as representative. For this special examination secretion from cases of mastitis due to the various streptococcus groups was plated and attention given to plates showing colonies apparently of the same kind. In this way, on each of 32 occasions, three to eleven colonies were tested and practically without exception their behaviour was similar serologically. In certain cases of infection with Group II streptococci there was, however, a notable difference in the agglutinability of the various colony strains.

PART II.

The only previous work on the serological characters of streptococci from mastitis secretion appears to have been done by Jones (1918), who applied the agglutination test to 41 strains of non-hæmolytic streptococci and to 29 strains of hæmolytic streptococci. All except one of the non-hæmolytics conformed in general characters to the streptococci which are the most common cause of chronic bovine mastitis. No absorption tests were made.

A serum prepared in a cow with one of the non-hæmolytic strains agglutinated all, but the titre varied from 1/20,000 with the homologous strain and with four others to less than 1/2,000 with ten strains. At this dilution one strain which was definitely distinguished by fermentation tests was positive. The significance of these differences is not clear, but they cannot altogether be referred to a difference in the stability of the suspensions

because all were described as not tending to precipitate during the 24 hours incubation period used. The results suggest that different types may have been present. Five hæmolytic strains were tested with this serum and were not agglutinated at 1/100. A serum prepared in a rabbit with a hæmolytic strain agglutinated the homologous strain and 13 others hæmolytic strains to 1/1,000, 19 others to 1/500, and five strains to less than 1/200. Non-hæmolytic strains were apparently not tested with this serum.

Source of Strains.

(*a*) *Clinical Cases of Mastitis.* This series contains 110 strains, from different quarters and in nearly all instances from different cows. About half of them were received from veterinary surgeons practising in widely separated areas, while the remainder were selected during the periodical examination of nine herds containing about 500 cows. No attempt has been made in this work to estimate the prevalence of the different serological types, though it is obvious that those of Group I are the most widespread.

(*b*) *Non-clinical Cases or Normal Udders.* This series contains 50 strains collected from the herds already mentioned, of which 25 were from milk in which definite signs of abnormality were detectable on laboratory examination only, and 25 from milk which at the time was normal.

There were also examined eight strains isolated in Germany by Dr. M. Seelemann, to whom I am indebted for forwarding them.

Serological Types.

A summary of the results with these strains is given in Tables II and III. It will be seen that Group I contains both hæmolytic and non-hæmolytic streptococci and Groups II and III non-hæmolytic strains only.

TABLE II.

One Hundred and Ten Strains from the Secretion of Cases of Severe Mastitis.

Group and Serological Type.	*Hæmolytic Strains (43).*		*Non-hæmolytic Strains (67).*	
	No. in Group.	*No. of each Type.*	*No. in Group.*	*No. of each Type.*
Ia	43	26	29	28
b		5		1
c		12		—
IIa	—	—	26	12
b		—		9
c		—		2
group		—		3
IIIa	—	—	12	3
b		—		1
c		—		3
group		—		2
unclassified		—		3

TABLE III.

FIFTY STRAINS FROM MILD CASES OF MASTITIS, ONLY DETECTED BY LABORATORY EXAMINATION, OR FROM NORMAL MILK.

Group and Serological Type.	*Totals.*		*Altered Milk.*		*Normal Milk.*	
	No. in Group.	*No. of each Type.*	*No. in Group.*	*No. of each Type.*	*No. in Group.*	*No. of each Type.*
Ia	19	7	10	3	9	4
b		—		—		—
c		12		7		5
IIa	3	1	2	—	1	1
b		1		1		—
c		1		1		—
group		—		—		—
IIIa...	28	2	13	1	15	1
b...		—		—		—
c...		4		1		3
unclassified ...		22		11		11
Totals ...	50 strains		25 strains		25 strains	

Groups II and III contained only non-hæmolytic strains, Group Ic only hæmolytic strains.

Most of the strains reported here have at some time been tested with at least one serum of each type ; many with several sera and on many occasions. It has gradually become clear, however, that a definite reaction with one type of serum indicates that a reaction will be obtained with any other serum of that type and with no other. In some cases, therefore, a strain which had already been referred to one type was not tested with new sera.

Group I.

The general characters of this group, which included both hæmolytic and non-hæmolytic strains, correspond to those of the streptococcus which is the common cause of chronic bovine mastitis, (the so-called *S. mastiditis contagiosæ* or *S. agalactiæ*) and have been described in two previous publications from this Institute (1929, 1932).

The group contains 91 strains : 55 produced β zones in ox-blood agar plates ; 36 gave an α or γ appearance. Serologically they were of three kinds.

Type A.

Sixty-one strains were of this type, including 54 from clinical cases, 26 being hæmolytic and 35 non-hæmolytic. By serological tests the hæmolytic and non-hæmolytic strains were similar, absorption even with graded doses revealing no difference. Table IV gives examples.

Satisfactory sera were obtained with ten strains, of which three were hæmolytic and seven non-hæmolytic. Seven sera were of sufficiently high value for precipitation tests. All would probably have been suitable for agglutination, but three were not available when this technique was first used. The titre varied from 1/640 to 1/40.

The strains used in producing these sera were entirely similar, and distinct from all others by reciprocal precipitation, agglutination and absorption tests.

Twenty-one of the 160 strains used in this work, which by agglutination gave a negative result with Ia sera, were not further tested with sera of this type.

TABLE IV.

PRECIPITIN-ABSORPTION TESTS OF TYPE Ia SERA WITH VARIOUS STRAINS.

Serum Absorbed.	*Precipitate with Unabsorbed Serum.*	*Absorbing Strain.*	*Type.*	*Amount of Absorbing Strain in c.c. and result.*			
				0·2	0·1	0·05	0·25
S57 (Ia, γ)... ...		S57	Ia (γ)	—	—	½	1
1·0 c.c. diluted with equal		S79	Ia (α)	—	—	½	1
vols. of saline ; 0·4 c.c.	1½	S6	Ia (β)	—	—	tr.	½
of mixture used in final		S39	Ia (β)	—	—	½	1
test.		S84	Ib (β)	2	/	/	/
S39 (Ia, β)	2	S81	Ic (β)	2	/	/	/
as above.		S79	Ia (γ)	—	—	tr.	1
		S39	Ia (β)	—	—	½	1½
S57 (Ia, γ)		S32	IIa (α)	2	/	/	/
0·50 c.c. diluted with one		S52	II group (α)	2	/	/	/
or two equal vols. of		N13	IIb (α)	2	/	/	/
saline ; equivalent of	2	S54	IIc (α)	2	/	/	/
0·25 c.c. of whole serum		S51	IIIa (α)	1½	/	/	/
used in final test.							

One strain, S37, was exceptional in that, although it was agglutinated nearly to titre by two Ia sera and its serum agglutinated Ia strains to about half titre, tests by precipitation and the corresponding absorption did not indicate the same similarity.

The results with all other strains were unequivocal, and showed that type Ia was a common and well-differentiated type, and this has been amply confirmed by more recent work.

Type B.

This contains 6 strains of which 1 was non-hæmolytic.

Satisfactory sera were obtained from three strains, S67, 77 and 84. Examination of these three sera by all methods showed that the strains concerned were alike and distinct from all other types. Sera S84 and 77 were chiefly used ; they have good precipitates with the corresponding extracts and had an agglutination titre of 1/320 and 1/160 respectively.

Type C.

This type contains 24 strains, all of which were hæmolytic. A serum prepared with one, S81, agglutinated all to a titre of 1/640 to 1/1280 ; absorption tests confirmed the specific nature of the reactions. Serum S81 also gave satisfactory precipitates with homologous extracts but none with extracts of 83 strains, representing all other types, which were tested with it. Precipitation tests with serum S81 were not carried out with the remaining 23 strains of this type because they were not met with until late in the work, when only a small amount of serum was available. Precipitation and agglutination tests with specific sera of other types were, however, negative.

Soon after the commencement of this work it was found that six strains, received from Dr. M. Seelemann, of Kiel, and regarded by him as typical of the mastitis streptococci prevalent in Germany (*S. agalactiæ*), though similar to Group Ia in biochemical characters, were not similar serologically. Recent examination of three further strains from this source has shown that one, which was labelled " typical," is again serologically different from Group I, type *a*, but corresponds exactly to type *b*—a type which has not occurred frequently in the series of strains now examined. On the other hand, the remaining two strains, which were labelled " atypical " correspond exactly to the streptococcus (Ia) which was the predominant serological type met with in the present series.

Group II.

This group contained 29 non-hæmolytic strains, of which 26 were from clinical cases of mastitis. They were characterised by growth and reduction in methylene blue milk, a high limiting *p*H in sugar media, and inability to utilise salicin. Their general characters have recently been fully described (Edwards, 1932).

Considered as a whole these 29 strains can be viewed as a serological group, because they are directly or indirectly connected antigenically, but there are two well-marked types containing 13 and 10 strains respectively, whilst three other strains are alike and three show group characters. These facts are illustrated in Table V, which includes representatives of all types tested with sera made group but not type specific. It gives the precipitation results of the Group II strains of the series named N_1—N_{40} and also those of two other strains tested at a different date. Tests for absorption of agglutinins are also included in the case of types *a* and *b*, and show results which are parallel with those of the precipitin tests. In the case of type *c* and the group strains, a full series of absorption tests has not been done, but, from the results of experiments carried out since this paper was written, in which sera of all four kinds were absorbed with various doses and tested against suspensions of each kind, it may be said that, whereas types *a* and *b* are entirely different, types *a* and *c* have an antigen in common. It was also clear that the group strain S52 not only possesses antigens in common with each type, but, in

TABLE V

PRECIPITATION AND AGGLUTININ-ABSORPTION TESTS OF GROUP II STRAINS OF SERIES N1 TO N40 AND OF S52, 54 AND 66.

	Precipitation tests.								Agglutinin-absorption tests.		Type of strains under test.
Sera.	*S*57	*N*18	*S*84	*S*32	*S*52	*N*3	*N*13	*S*54	*S*51	*S*32	*N*13	
Group and Type	Ia	Ia	Ib	IIa	II group	IIb	IIb	IIc	IIIa	IIa	IIb	
Homologous Control ...	2	1	2	2	2	1	2	1	1(c)	—	—	
N3	—	—	—	—	tr.	1	2	—	—	640	—	IIb
N4	—	—	—	3	½	—	—	—	—	tr. 40	40	IIa
N7	—	—	—	2	tr.	—	—	—	—	—	40	IIa
N8	—	—	—	3	½	—	—	—	—	—	40	IIa
N12	—	—	—	—	½	1	2	1	—	640	tr. 10	? IIb
N13	—	—	—	—	½	1½	2	—	—	640	—	IIb
N14	—	—	—	2	½	—	—	—	—	±40	40	IIa
N15	—	—	—	3	½	—	—	—	—	—	40	IIa

N21	—	—	tr.	2	3	tr.	1	4	—	320	40	II group
N23	—	—	—	2	tr.	—	—	—	—	—	40	IIa
N32	—	—	—	2	½	—	—	—	—	—	40	IIa
N33	—	—	—	—	tr.	2	2	—	—	640	—	IIb
N35	—	—	—	—	tr.	2	2	—	—	640	±10	IIb
N37	—	—	—	1½	1	—	½	1	—	320	20	II group
N39	—	—	—	2	tr.	—	—	—	—	—	40	IIa
N40	—	—	—	2	tr.	—	—	—	—	±40	40	IIa
S52	—	—	/	1	3	/	tr.	2	—	320	±10	II group
S54	—	—	—	—	1½	—	—	1	—	160	40	IIc
S66	—	—	/	—	2	/	—	1	—	80	40	IIc

In the precipitation tests, 0·5 of each extract was mixed with the amount of each serum found suitable with the homologous extract, incubated at 53° C. for 1½ hours and read after standing overnight. For the absorption tests, two minimal absorption doses were used. Serum S32 had a titre of 1/640 and was used at 1/40. Serum N13 had a titre of 1/40 and was used at 1/10. The first three lines indicate the sera used, their type, and the precipitate with, or absorption by, the homologous strain. The strains under test are indicated in the left-hand column. The figures in the precipitation tests, 4, 3 . . tr., indicate amounts of precipitate, — = none, / = no test. The figures in the absorption tests are the titres for the homologous suspension after absorption.

addition, judging from the results of absorptions of serum S52 with all types, separately or at the same time, a certain amount of antigen qualitatively different and peculiar to itself.

Type A.

Twelve strains were of this type, of which nine are shown in Table V. It will be seen these strains gave a full precipitate with the Group II type *a* serum, a small precipitate with the Group II group serum (S52) and none with either of the Group II type *b* sera, the Group II type *c* serum, or sera of any other type.

In order to be sure that absence of reaction with certain extracts was not due to unsuitable proportions of antigen and antibody, retests were made with various volumes of extract but still with a negative result.

At various times useful sera have been obtained with three strains of type *a*, S12, S32 and N4, their agglutination titres being 1/1280, 1/640 and 1/80 respectively. By all direct and absorption tests the strains were similar and distinct from other types.

Type B.

Ten strains were of this type, of which five are shown in Table V. They reacted fully with type *b* sera, slightly with the group serum S52, and, except in the case of N12, with no other. Three useful sera (N3, N13 and N33) have been obtained and proved similar by all methods.

It should be remarked here that two of the sera, although quite satisfactory in precipitation tests, as indicated in Table V, did not agglutinate the serum strains to above 1/40, nor any other of the strains which they picked out as similar by precipitation ; strains which moreover were shown to be similar by other tests and sera. The reason for this is not obvious. N33 serum was not available when this was learnt, but fortunately it was already known that the serum prepared with the group strain S52 could be rendered specific for Type *b* strains by suitable absorption.

Type C.

Three strains were of this type, of which two S54 and 66, are included in Table V. By precipitation they gave no reaction with type *b* sera, and never more than a trace with type *a* sera, but a typical precipitate was produced with the serum from the group strain S52. Suspensions of these three strains were agglutinated by S52 serum, not by type *b* sera, but to about half titre by the only type *a* serum with which they were tested.

A serum prepared with S54 gave a positive precipitation result with each type *c* strain, but none with strains of types *a* or *b*. The same serum agglutinated each type *c* strain to about 1/160. It also agglutinated to about half titre the type *a* strains, and two of the group strains about to be considered, but S52 was only agglutinated to 1/40.

Group II Group.

Three strains were found to give some reaction by both precipitation and agglutination with sera of all types. To one of these, S52, with which a serum was produced, reference has already been made. Extracts of this strain containing a normal amount of antigenic material, as judged by the precipitates obtained when mixed with its own serum, gave a normal precipitate with a type *c* serum, a small though constant precipitate with type *a* sera, and a smaller amount with type *b* sera, the amount with a type *a* serum being not more than half that obtained with the homologous extract.

Serum S52 gave a small precipitate with all Group II extracts, though a normal one with its own. This is also exemplified in Table V. Absorption experiments confirmed this evidence that S52 contained all the antigens of the group though in relatively small amount. Thus, serum S12 (type *a*) was completely absorbed by one treatment with a certain dose of the two typical type *a* strains S12 and S32, but only about half the antibodies were removed by S52. A second absorption removed all but a trace. Other experiments indicated a similar ability of S52 to remove antibodies from sera of all other types within Group II, the amount of antibody removed varying in relation to the absorbing dose.

Serum S52, rendered group but not type specific, agglutinated all strains of Group II, but those of type *a* less quickly at any given dilution, and to about half the titre at which type *b* or *c* suspensions were agglutinated. By absorption it was found that each of three type *a* strains removed all type *a* agglutinins, but had no effect on those of *b*. Conversely, a type *b* strain removed *b* agglutinins and had no effect on those of *a*. One of the type *a* strains was used in three absorbing amounts, one of which was four times the minimal absorbing dose of the homologous strain, but still no absorption of type *b* antibodies occurred. The antigens of types *a* and *b* are thus qualitively different, but both are present in strain S52.

Suspensions of S52 were agglutinated by the homologous serum to about 1/640, but not above 1/10 by any type *a* serum, and, rather surprisingly, not above 1/40 by the type *c* (S54) serum.

Further strains of types *a* and *b* have frequently been isolated during routine tests, and there is some evidence that still other types may occur within this group, which is being further studied.

Group III.

The term Group III has been used by us to denote non-hæmolytic strains of mastitis streptococci, whose characters included fermentation of mannite, inulin and salicin, and growth and reduction in methylene blue milk. Their characters have been fully described recently by Edwards (1932). From a serological viewpoint group likeness was not marked.

Of 40 strains, 25 have not been classified, whilst 15 fall into three types, called *a*, *b* and *c* respectively. Satisfactory agglutinating sera,

obtained with three strains S51, N20 and N29 proved to be diverse.

Of the 15 strains, five were of type *a* (S51), one was of type *b* (N20) and seven of type *c* (N29), whilst two strains were agglutinated to about half titre by sera S51 and N20. Strains of all these types have been met with since this work was completed.

The titre was in each case low, about 1/80, and no precipitate was obtained when 1·0 c.c. or less was mixed with 0·5 c.c. of homologous extract, except in the case of S51, when it was transparent, disc-like and clearly of a carbohydrate nature. Sera of other types, which agglutinate at 1/80, have always given some precipitate with homologous extracts. There is, therefore, some reason to believe that the strains concerned here are deficient in the type-specific protein ; and it seems probable from other tests that all the strains of this group are likewise deficient.

Although the sera of this group were of low titre, the results obtained were clear-cut, agglutination being particularly rapid and coarse at 1/10 to 1/40 in the case of types *b* and *c* sera. Suspensions of all strains of this group were perfectly stable. Two sera, obtained with each type, showed equal specificity. Agglutinin-absorption tests gave clear results and confirmed the diverse nature of the type sera.

Discussion.

It should be emphasised that the results reported are those of a preliminary survey of the serological characters of mastitis streptococci and of the relations of these to hæmolysis and biochemical characters in general, and that they are not intended to indicate the relative prevalence of the several groups or types in udder secretion as a whole or in samples of various characters. It is also obvious that other types may exist in Groups I and II as well as in Group III. There seems no doubt, however, that the various types of Group I are responsible for by far the larger number of cases of chronic bovine mastitis.

In previous reports we have described two groups of hæmolytic streptococci and three groups (I, II and III) of non-hæmolytic streptococci encountered in mastitis. The larger hæmolytic group was on other grounds indistinguishable from the non-hæmolytic Group I. This similarity, coupled with the finding reported in this paper that, as regards most strains, they are serologically indistinguishable, and with the fact that the mastitis caused is of a similar nature, makes it justifiable to include them in one group. Group I may, therefore, be hæmolytic or non-hæmolytic. So far, however, all strains of Group I, type *c*, have been hæmolytic. The groups I, II and III, which have already been distinguished on general and biochemical grounds, are distinct serologically and, further, each group contains several types which, though similar biochemically

are serologically diverse. The serological relationship between groups and types has already been discussed in Part I.

The finding that certain strains of *S. agalactiæ* from Germany fell into serological types which occur in this country and that the strains labelled " typical " were of a type not commonly encountered in the present series, whereas those labelled " atypical " were of the most frequent serological type, is not without interest.

It will have been observed that in Group I serological tests revealed no qualitative or quantitative distinction between strains which were hæmolytic in ox-blood agar plates and those which were non-hæmolytic. This should prove to be a valuable means of studying a change of hæmolytic properties as defined, *in vivo* and *in vitro*. Certain observations made in this work have strongly suggested that such a change may occur, but consideration of these must be reserved until more evidence has accumulated. Reference should also be made to the hæmolytic characters of the strains of Group I, type *b*. On looking up the files of these strains it was found that they were all similar and somewhat peculiar in regard to hæmolysis. After 24 or 48 hours incubation a large part of the colonies were surrounded by a narrow but clear zone of hæmolysis outside which was produced a greenish ring similar to that found immediately around the colony of an α strain. Outside this ring of fixed cells was another in which hæmolysis was again complete. This appearance does not exactly correspond to Brown's description of an α_1 nor in fact to any of Brown's categories. There was, moreover, another character which precluded the placing of these amongst the α group, *viz.*, the marked lysin production in fluid media. It should be added that in strains of no other type was this strong double-ringed tendency observed. It will have been noted that, in contrast to Group I, Groups II and III contained only non-hæmolytic strains.

The serological composition of the various groups was also different. All the Group I strains of this series fell into three types which showed only slight group similarity. In Group II, group characters were marked from the serological viewpoint and all strains had antigens in common with one of the three type strains. Two of the types showed no likeness, but there were some strains which had a considerable amount of antigen in common with more than one type. Group III on the other hand appeared to be more heterogenous ; more than half the strains had no likeness whatever to any of the three strains with which type sera were prepared.

Strains which are biochemically and in hæmolytic character of the nature of *S. pyogenes* of direct human origin, but which have been isolated from cases of bovine mastitis, strains of *S. lactis* and of *S. cremoris*, and a group of non-pathogenic streptococci which are frequently encountered in carefully taken samples, will be dealt with in subsequent reports. It may be said, however, that none of these strains have shown any serological likeness to the mastitis streptococci described here.

Summary.

1. It has been shown that each of the Groups I, II and III into which streptococci from cases of bovine mastitis can be arranged on cultural and biochemical grounds, can be further divided by means of serological methods.

2. The methods used were precipitation at 50° to 55° C., using extracts prepared with hot M/20 HCl. in saline, agglutination by means of a rapid macroscopic slide technique at room temperature and the corresponding absorption tests.

Immune sera were rendered type-specific by means of preliminary absorption.

3. The precipitation test was applicable to all types of streptococci examined, except those of Group III, which appeared to be deficient in type-specific protein.

4. The slide agglutination technique gave easily read and reproducible results, and by the methods described both direct agglutination and agglutinin-absorption tests were carried out quickly with minimal amounts of serum and suspension.

Suspensions which were not sufficiently stable for tube agglutination tests at higher temperatures were in general satisfactory for the slide test. A few strains always gave very granular suspensions and could only be dealt with by agglutinin-absorption or by precipitation.

5. Except in the case of Group III, the results obtained by precipitation and agglutination were parallel. In Group II, however, certain minor differences were observed.

6. One hundred and sixty strains from different quarters of 151 animals have been examined and among these nine serological types have been recognised ; 110 strains were from clinical cases of mastitis.

Group I, 91 strains. In general characters these correspond with the group of streptococci which are the cause of chronic bovine mastitis (the so-called *S. mastiditis* or *S. agalactiæ*) ; 61 strains were of type Ia, 6 of type Ib, and 24 of type Ic.

Group II, 29 strains, of which twelve were of type IIa, ten of type IIb, and three of type IIc. Three strains contained antigens belonging to each of these types.

Group III, 40 strains, of which 15 were classified, *viz.*, type IIIa (five strains) ; type IIIb (one strain) ; type IIIc (seven strains) and two strains which gave partial reactions with sera of both types *a* and *b* ; 25 strains gave a negative result with all sera.

7. Of 91 strains in Group I, 55 were hæmolytic (β) and 36 non-hæmolytic (α or γ) in ox-blood agar plates. Both hæmolytic and non-hæmolytic forms were encountered in types *a* and *b* of this group and were found to be alike by direct precipitation and agglutination test and by absorption with graded amounts of organisms. So far, all strains of type *c* have been hæmolytic.

8. Except in regard to hæmolysis, strains of the same serological type were alike biochemically. Strains which behaved similarly in biochemical tests, *i.e.*, strains of the same group, did not necessarily belong to the same serological type. Strains of different biochemical groups were never alike serologically.

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GAS GANGRENE INFECTIONS OF SHEEP: PASSIVE IMMUNISATION.

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Investigations on the ætiology of gas gangrene of sheep were described in a previous paper* and *B. chauvœi* was found to be the common causal micro-organism against which prophylactic measures should be taken. During the 1932 lambing season further cases have been studied, and the results confirm the observations previously made.

The bacteriology of the cases examined this year is summarised below. The remainder of the paper is concerned with passive immunisation against *B. chauvœi* by methods which appear to be of practicable application in the district where the disease has been encountered, *viz.*, the Romney Marsh.

Summary of Cases in 1932.

Altogether 19 cases have been investigated. Material for bacteriological examination was collected personally and in 18 instances post-mortem examinations were made. The bacteria were recovered either from affected muscle tissue or from the subcutaneous or intramuscular infiltrations.

Eleven cases of post-parturient gas gangrene in the ewe were obtained. *B. chauvœi* was the only pathogenic micro-organism recovered from seven of these, but *B. chauvœi* and *V. septique* were isolated in three instances, and in one instance *B. chauvœi* and a bacillus which was probably *B. welchii.* In the latter instance *B. welchii* was not observed in smears made from the original material; these indicated a *B. chauvœi* infection only. A *B. chauvœi* infection was found in a wether sheep which contracted gas gangrene a few days after the fleece had been clipped from around the hind quarters. Seven cases of the disease in lambs following docking or castration and docking, showed in five instances a *B. chauvœi* infection, while from the remaining two both *B. chauvœi* and *V. septique* were isolated.

In the previous paper the significance of the recovery of *V. septique* was discussed, and it was emphasised that, had *V. septique* and *B. chauvœi* both been present in the original material, the former, owing to its more vigorous growth and greater pathogenicity for laboratory animals, would mask the presence of the latter when simple cultural means or animal inoculations were used to isolate and identify the organisms. A consideration of the evidence then obtained indicated the probability of *V. septique* being a post-mortem or secondary invader and not the primary cause of the disease.

* Roberts, R. S., and McEwen, A. D. Jl. Comp. Path & Ther., **44**, 180.

When gas gangrene is experimentally produced in sheep by intramuscular inoculation with either *V. septique* or *B. chauvœi* the macroscopic local lesions are very similar, but in the *V. septique* infection there is generally congestion of the mucosa of the abomasum and of parts of the small intestine, while in the *B. chauvœi* infection these are rarely observed. Therefore, in field cases post-mortem examinations may assist as a guide to the differentiation of the causal micro-organism should this be either *V. septique* or *B. chauvœi*. Further, microscopical examinations of smears from local lesions are of differential aid, as *B. chauvœi* appears as a more slender and elegant micro-organism than *V. septique*, and these microscopical differences are generally apparent to an examiner familiar with the appearance of both micro-organisms when growing in muscle tissue. In all the nineteen cases examined during the past season the evidence gathered from post-mortem examinations and the examinations of smears from local lesions indicated a *B. chauvœi* infection, and it is considered that when *V. septique* was isolated it had been present in the body as a secondary invader and not as the causal micro-organism.

The immunisation of cattle against *B. chauvœi* infection is generally successful and, a priori, it would appear that sheep could likewise be immunised against ovine strains of the bacillus, but were it possible to immunise sheep successfully it is doubtful whether it would be extensively practised in the area where we have encountered the disease. In all classes of sheep the disease is most irregular in its appearance, and farmers frankly admit that even if the disease could be controlled by the inoculation of the whole flock they would rather take the risk of a number of animals contracting the disease than incur the labour and expense of inoculation.

Parturient ewes receiving assistance at lambing suffer more from gas gangrene than do other classes of sheep. If it were possible for the shepherd to protect these animals by inoculating them with immune serum either before or immediately after giving assistance a practical method for saving considerable numbers of animals would be available, and experiments have therefore been undertaken with a view to ascertaining if this could be accomplished. Controlled experiments have demonstrated the value of immune serum in preventing the appearance of an inoculation gas gangrene.

General Methods.

Infection with *B. chauvœi* was produced by inoculating the experimental animals with quantities of diffusion shell broth culture. The cultures used were obtained from a virulent strain of the organism recovered from a sheep which had died from gas gangrene in 1930, and the virulence of the strain was maintained by recovering the micro-organism from time to time from the muscle lesions of inoculated control sheep. After incubation the cultures to be used for infective inoculations were kept in the ice chest, where their virulence remained at an approximately constant level for several weeks, during which time they were available as a supply of infective material.

For each experiment as detailed in the protocols all inoculations were made on one and the same date unless the contrary is expressly stated.

When mice were used the culture was inoculated subcutaneously, and with guinea-pigs and sheep inoculations were made intramuscularly.

In attempts to estimate the lethal dose for mice the animals were employed in groups each containing three animals, all members of a group being inoculated with the same quantity of culture. The amounts of culture inoculated per mouse per group were 0·01 c.c., 0·05 c.c., 0·1 c.c., 0·3 c.c., and 0·4 c.c. Fatalities occurred in each group except that in which each mouse received 0·01 c.c., but the mortality was not complete in any group and therefore in further experiments with these animals a relatively large dose of culture was inoculated and by using a number of animals an effort was made to obtain data of some value.

In the case of the guinea-pig 0·5 c.c. of culture was generally fatal and this quantity or 1 c.c. was used in immunity experiments.

With the first experiments on sheep an infective dose of culture of 0·5 c.c. was given, but in subsequent experiments the quantity was reduced to 0·25 c.c. or 0·0625 c.c., the smaller amount almost invariably being lethal.

In 1931 a polyvalent horse serum was prepared, a mixture of equal parts of formalinised culture of four strains of *B. chauvœi* isolated from sheep being used as antigen. The strain used for the infective inoculation was not included in this polyvalent antigen. The following year a further supply of serum was required. By this time the strain used for infective inoculations was the only one which had maintained its full virulence and capacity of vigorous growth

TABLE I.

SERUM AND CULTURE MIXED BEFORE INOCULATION.

a.	*No. of Guinea Pigs.*	*Serum*, 1931.	*Culture.*	*Result.*
	2	0·1 c.c.	0·5 c.c.	L.L.
	4	0·05 c.c.	0·5 c.c.	L.L.L.L.
	6	———	0·5 c.c.	D.D.D.D.D.D.
———		*Serum*, 1932.		
b.	3	0·05 c.c.	1 c.c.	L.L.L.
———	3	0·025 c.c.	1 c.c.	L.L.L.
	3	———	1 c.c.	D.D.D.
	2	0·016 c.c.	1 c.c.	L.L.
	2	0·0125 c.c.	1 c.c.	L.L.
	2	———	1 c.c.	D.D.
	2	0·01 c.c.	1 c.c.	L.L.
	2	0·008 c.c.	1 c.c.	L.L.
	1	———	1 c.c.	Extensive lesions but recovered.

Section *b* of this experiment was carried out in three parts on three consecutive days, the same culture being used each day.

in broth medium, and formalinised cultures of it were inoculated into the previously immunised horse and a further supply of serum drawn in 1932.

Preliminary demonstrations of antibody in the sera were obtained from guinea-pigs inoculated with mixtures of culture and varying quantities of sera which had been allowed to stand at room temperature for one hour. In 1931 the preliminary test was made before completion of the immunising process.

Using methods similar to those employed above, a comparison was made between the serum prepared in 1931 against ovine strains of the bacillus and a commercial serum prepared presumably against a bovine strain of the micro-organism. The results are given in Table II and indicate the superiority of the 1931 serum.

TABLE II.

No. of Guinea Pigs.	*Serum.*	*Amount of Serum.*	*Culture.*	*Result.*
3	1931	0·2 c.c.	1 c.c.	All lived.
3	Commercial	0·2 c.c.	1 c.c.	Two died.
7	1931	0·1 c.c.	1 c.c.	One died.
7	Commercial	0·1 c.c.	1 c.c.	Five died.
6	1931	0·05 c.c.	1 c.c.	Five died.
6	Commercial	0·05 c.c.	1 c.c.	All died.
3	1931	0·025 c.c.	1 c.c.	All died.
3	Commercial	0·025 c.c.	1 c.c.	All died.
2	Normal horse serum	0·2 c.c.	1 c.c.	All died.
2	Do.	0·1 c.c.	1 c.c.	All died.
2	Do.	0·05 c.c.	1 c.c.	All died.
4	———	———	1 c.c.	All died.

A similar experiment was carried out in mice but without showing any decided difference in the neutralising powers of the two sera.

TABLE III.

No. of Mice.	*Serum.*	*Amount of Serum.*	*Culture.*	*Result.*
3	1931	0·05 c.c.	0·5 c.c.	All lived.
3	Commercial	0·05 c.c.	0·5 c.c.	All lived.
3	1931	0·025 c.c.	0·5 c.c.	Two died.
3	Commercial	0·025 c.c.	0·5 c.c.	One died.
3	1931	0·0125 c.c.	0·5 c.c.	All died.
3	Commercial	0·0125 c.c.	0·5 c.c.	All died.

As these and other experiments reported in the following pages indicated that the serum prepared against ovine strains of the bacillus was of greater potency in neutralising the pathogenicity of ovine strains of *B. chauvœi*, the greater portion of the experimental work was carried out with sera prepared against ovine strains of the organism and only these sera were used in the field.

Passive Immunity Experiments on Laboratory Animals. The simultaneous Inoculation of Serum and Culture.

Guinea-pigs and mice were inoculated with culture and at another part of the body with serum. When a sufficiently large dose of serum was administered to guinea-pigs the inoculation disease did not develop. Mice were not protected by a comparatively small amount of serum inoculated subcutaneously, but when the serum was given by the intravenous route an appreciable protection was evidenced.

TABLE IV.

No. of Guinea Pigs.	*Culture.*	*Serum* 1931.	*Result.*
3	0·5 c.c. intramuscularly	3 c.c. subcutaneously	D.L.L.
3	,, ,,	6 c.c. ,,	L.L.L.
3	,, ,,	None	D.D.D.
No. of Mice.			
6	0·35 c.c. subcutaneously	0·5 c.c. ,,	D.D.D.D.D.D.
6	,, ,,	1 c.c. ,,	D.D.D.D.L.L.
6	,, ,,	None	D.D.D.D.L.L.
6	,, ,,	0·5 c.c. intravenously	D.L.L.L.L.L.
6	,, ,,	None	D.D.D.D.D.D.

With both species of animals the amount of culture inoculated was large, and when the comparative size of the animals is considered it is evident that the amount inoculated into the mice was relatively much larger than the quantity given to guinea-pigs. In view of this it is not surprising that the comparatively small quantity of serum inoculated subcutaneously into mice failed to confer protection. The success attendant upon the intravenous inoculation of serum into mice was, however, encouraging.

The Effect of a Time Interval between the Inoculation of Culture and the Inoculation of Serum.

Guinea-pigs and mice were inoculated with culture, and then and also two hours and four hours later groups of these animals received serum. In the case of mice serum was withheld until the lapse of even longer intervals of time. On the whole the results indicated that the delayed inoculation of serum gave the animals some protection.

From the Table V it will be seen that the guinea-pigs behaved in an irregular manner, though possibly some protection was conferred by serum even when this had been withheld for four hours after the infective inoculation had been made. Mice also gave somewhat irregular results, mortality not being complete among the control animals. The experiment on mice was carried out in two parts on two consecutive days. On the second day the infective dose was increased in amount in the hope that the control animals would succumb, and on the same day in attempting to make the experiment more critical the amount of serum inoculated into the mice eight hours

TABLE V.

No. of Guinea Pigs.	*Culture.*	*Interval.*	*Serum 1931.*	*Result.*
4	0·5 c.c intramuscularly	Simultaneously	5 c.c subcutaneously	D.L.L.L.
4	,, ,,	2 hrs. leter	,, ,,	D.D.D.L.
4	,, ,,	4 ,, ,,	,, ,,	D.D.L.L.
4	,, ,,	———	None	D.D.D.D.
No. of Mice.				
4	0·3 c.c. subcutaneously	Simultaneously	1 c.c. intravenously	L.L.L.L.
4	,, ,,	2 hrs. later	,, ,,	L.L.L.L.
4	,, ,,	4 ,, ,,	,, ,,	L.L.L.L.
4	,, ,,	6 ,, ,,	,, ,,	L.L.L.L.
4	,, ,,	———	None	D.D.L.L.
4	0.5 c.c. ,,	8 hrs. later	0·5 c.c. intravenously	D.D.D.L.
4	,, ,,	12 ,, ,,	1 c.c. ,,	D.L.L.L.
4	,, ,,	16 ,, ,,	,, ,,	D.D.D.L.
4	,, ,,	After symptoms had appeared.	,, ,,	D.D.D.D.
4	,, ,,	———	None	D.D.L.L.

after injection was reduced, but as some of these serum-treated animals were commencing to show symptoms of illness by the time the animals in the next group were due to receive serum a reversion was made to the larger quantity of serum.

The experimental work was next extended to sheep, the animal which we hoped might be protected by the inoculation of immune serum in the field.

Passive Immunity of Sheep.

As a preliminary experiment a sheep was inoculated with 20 c.c. of " ovine serum 1931 " and 48 hours later this sheep and a control were each inoculated intramuscularly with 0·5 c.c. of culture. The animal inoculated with serum remained well but the control sheep died from an acute gas gangrene infection within 16 hours.

Culture and serum were next inoculated simultaneously and in the first experiment " ovine serum 1931 " and the commercial serum were employed. The first portion of the experiment was designed in the hope of receiving from it data regarding the comparative value of the two sera. An infective inoculation of 0·5 c.c. of culture was used and against this large infective dose varying quantities of the two sera were tested.

Despite the very irregular results of this experiment, it is possible that some protection had been afforded to certain of the animals by the serum, as it was considered unlikely that any animal receiving 0·5 c.c. of the culture intramuscularly would have survived had serum not been given. But within the range of the amounts of serum given there appeared no optimum quantity and neither serum was clearly superior to the other.

Table VI.

No. of Sheep.	*Culture Inoculated Intramuscularly.*	*Serum.*	*Result.*
1	0·5 c.c.	50 c.c. ovine serum, 1931 simultaneously subcutaneously	Survived.
2	,,	20 c.c. ,,	Died 24 hrs. later.
3	,,	10 c.c. ,,	,, ,, ,, ,,
4	,,	10 c.c. ,,	Survived.
5	,,	50 c.c. commercial serum simultaneously subcutaneously	Chloroformed in extremis 48 hrs. later.
6	,,	20 c.c. ,,	Survived.
7	,,	10 c.c. ,,	Died 24 hrs. later.
8	,,	10 c.c. ,,	,, ,, ,, ,,
9	,,	None	,, 20 ,, ,,
10	,,	,,	,, 40 ,, ,,

In the next step the test inoculation of culture was reduced in quantity and the experiment carried out in two parts. The value of a simultaneous subcutaneous inoculation of 20 c.c. of ovine serum 1931 was first tested, and as this gave encouraging results the experiment was repeated but augmented by a comparative test with the commercial serum.

Table VII.

1st Part. *Sheep.*	*Culture Inoculated Intramuscularly.*	*Serum.*	*Result.*
1	0·25 c.c.	20 c.c. ovine serum 1931 simultaneously subcutaneously	Survived.
2	,,	,,	,,
3	,,	None	Found dead 42 hrs. later.
4	,,	,,	,, ,, ,, ,, ,,
2nd Part.			
5	,,	20 c.c. ovine serum 1931 simultaneously subcutaneously	Died 4 days later.
6	,,	,,	Survived.
7	,,	20 c.c. commercial serum simultaneously subcutaneously	,,
8	,,	,, ,,	Died 21 hrs. later.
9	,,	None	,, ,, ,, ,,
10	,,	,,	,, 24 ,, ,,

From this experiment the value of the inoculation of ovine serum 1931 definitely appeared, three out of four sheep treated with serum surviving while all four control sheep died. Again, the fourth sheep inoculated with serum lived for a much longer period than did any of the controls. Of the two animals treated with commercial serum one survived.

The experiment was repeated with the infective inoculation of culture further reduced in amount. The same culture kept in cold store was used to furnish the infective inoculation in the experiments recorded in Tables VI, VII and VIII.

TABLE VIII.

Part I. Sheep.	*Culture Inoculated Intramuscularly.*	*Serum.*	*Result.*
1	0·0625 c.c.	20 c.c ovine serum, 1931 simultaneously subcutaneously	Survived.
2	,,	,, ,,	,,
3	,,	None	Died 24 hrs. later.
4	,,	,,	Found dead 40 hrs. later.
Part 2.			
5	,,	20 c.c. ovine serum, 1931 simultaneously subcutaneously	Survived.
6	,,	,, ,,	,,
7	,,	20 c.c. commercial serum simultaneously subcutaneously	,,
8	,,	,, ,,	,,
9	,,	None	Died 21 hrs. later.
10	,,	,,	Survived.

From this experiment the value of the serum was again demonstrated, as all six sheep receiving serum lived and three out of the four controls died.

It was now considered that, provided the lethal dose of culture was not excessive, the serum had a decidedly favourable influence in overcoming the infection, for when the results of the experiments are grouped together they show that nine out of twelve sheep receiving " ovine serum 1931 " lived, whereas nine out of ten controls died.

In the comparative tests of the " ovine serum 1931 " and the commercial serum, five out of eight sheep treated with the former and four out of eight treated with the latter lived. As the results of the comparative experiments on sheep and guinea-pigs slightly favoured the use of the ovine serum, further experimental work was confined to the ovine sera.

The Effect of a Time Interval Between the Inoculation of Culture and the administration of Serum.

In the following experiment serum was inoculated four hours after the inoculation of culture and the experiment was carried out in two parts. In the first part the amount of culture inoculated was 0·25 c.c., but both animals receiving serum and the control animals died of gas gangrene, and in the second part where the culture was reduced to 0·0625 c.c. the animals receiving serum survived but so did one of the two controls.

Table IX.

Part 1. *Sheep.*	*Culture Inoculated Intramuscularly.*	*Serum.*	*Result.*
1	0·25 c.c.	20 c.c. ovine serum, 1931 simultaneously subcutaneously	Died in 72 hrs.
2	,,	,, ,,	,, ,, 28 ,,
3	,,	None	,, ,, 21 ,,
4	,,	,,	,, ,, 24 ,,
Part 2.			
5	0·0625 c.c.	20 c.c. ovine serum, 1931 simultaneously subcutaneously	Survived.
6	,,	,, ,,	,,
7	,,	None	,,
8	,,	,,	Died in 21 hrs.

During the past lambing season a number of favourable results were reported from the fields when the administration of serum had been delayed until several hours after assistance had been given to ewes and the shepherd suspected them of having contracted a gas gangrene infection. Also the inoculation of serum into lambs already showing symptoms had apparently averted the disease. Comparatively large doses of serum had been inoculated into these ewes and lambs and in some cases two inoculations with an interval between had been made. Proof that the animals were infected with *B. chauvœi* was lacking, but it is likely that they were so infected because *B. chauvœi* infection had been diagnosed from the bacteriological examination of cadavers of incontact ewes which had not received serum and of two of the lambs which died shortly after inoculation with serum. The use of serum was not expected to give favourable results in lambs, as they would not be treated until symptoms were shown and the disease widely established. These favourable reports were obtained for the ovine serum drawn in 1932, for which our preliminary titration experiments indicated a high potency. This serum was therefore tested further for its capacity to neutralise or overcome an infection already established in the body. It will be seen from the Table X and the interpretation of the results that the subcutaneous or intravenous inoculation of 100 c.c. of serum given four hours after the inoculation of culture prevented the animals succumbing to a *B. chauvœi* infection and that the intravenous inoculation of large quantities of serum arrested the advanced symptoms of the disease and led to recovery.

The course of the infection in Sheep No. 2 was peculiar ; typical signs of an intramuscular inoculation infection with *B. chauvœi* were not seen, the inoculated limb showing neither tumefaction, œdema, nor discoloration. Between the eighteenth and thirty-sixth hours after the inoculation of culture the sheep remained lying down but did not show signs of acute pain. It was found dead in the morning of the third day and post-mortem examination revealed

TABLE X.

THE SAME CULTURE WAS USED FOR ALL THE INFECTIVE INOCULATIONS.

Sheep.	*Date of Inoculation.*	*Culture Inoculated Intra-muscularly.*	*Interval between Inoculation of Culture and Serum.*	*Serum.*	*Result.*
1	12.4.32	0·0625 c.c.	——	None	Died in 30 hrs.
2	13.4.32	,,	4 hrs.	100 c.c. subcutaneously	Died 3rd day. Atypical case, very mild local lesion but fœtus in uterus showed an acute infection with *B. chauvœi*.
3	14.4.32	,,	4 hrs.	100 c.c intravenously.	Survived.
4	14.4.32	,,	,,	,, ,,	,,
5	15.4.32	,,	,,	100 c.c. subcutaneously.	,,
6	15.4.32	,,	——	None	Died in 21 hrs.
7	18.4.32	,,	8 hrs.	100 c.c. intravenously.	Survived.
8	18.4.32	,,	,,	,, ,,	,,
9	20.4.32	,,	12 hrs. when symptoms warranted a fatal prognosis had serum not been given.	2 intravenous inoculations each of 100 c.c. at intervals of 2 hrs.	Killed 5 days after infection owing to debility following the removal of a very decomposed immature fœtus 36 hrs. after infection; only traces of retrogressive *B. chauvœi* infection found at post-mortem.
10	20.4.32	,,	,, ,,	,, ,,	Survived.

only a very slight intramuscular and subcutaneous œdema of the inoculated thigh ; no muscle lesions were found. The animal, however, was pregnant and the muscular and subcutaneous tissues of the intra-uterine fœtus showed extensive blackquarter or gas gangrene lesions. The integument was distended by gas and smears from the fœtal musculature showed very large numbers of bacteria morphologically identical with *B. chauvœi*. Only after prolonged search of smears made from the ewe's muscles at the seat of inoculation were micro-organisms recognised.

Sheep No. 9 was inoculated with serum twelve hours after the administration of an infective dose of culture and by this time it was registering a temperature of 105° F. and breathing was accelerated ; the inoculated limb was swollen and carried off the ground, and when disturbed the sheep fell and remained lying down on its side. At this time 100 c.c. of serum was given intravenously. Two hours later the temperature registered 106·4° F., and another inoculation of 100 c.c. of serum was made. By the sixteenth hour the temperature had dropped to 105·3° F. The animal was left for the night and

on the following morning, 36 hours after infection, a putrid discharge was found coming from the vulva. Examination showed a disintegrating, putrid, immature fœtus protruding into the vagina. This was removed in pieces. From the appearance of the fœtus it apparently had died some time before the commencement of the experiment. During the ensuing three days the ewe remained recumbent in an exceedingly weak condition, though eating and drinking when food and water were placed within reach. The condition of the animal remained stationary and on the fifth day it was considered advisable to slaughter her. At post-mortem examination the carcase was found to be poor and wasted, there was no involution of the uterus and this organ contained between 300 c.c. and 400 c.c. of thin, dark-coloured, putrid fluid. The inoculated limb was œdematous below the hock but elsewhere normal in size. Here and there in the depth of the thigh muscle were small dark areas or streaks—apparently all that remained of a gas gangrene or blackquarter muscle lesion. When thick smears were made from the dark coloured areas only one micro-organism was found per 15 to 20 fields of the microscope examined, and a growth of *B. chauvœi* was only obtained in minced meat broth medium when a piece of infected muscle the size of a filbert nut was inoculated. Where smaller pieces were inoculated no growth occurred, and a culture tube inoculated with 1·5 c.c. of the œdematous fluid from the lower part of the limb remained sterile.

Sheep No. 10 was lame and dull twelve hours after infection and registered a temperature of 102° F. Respirations were rapid and the animal allowed itself to be approached without moving. 100 c.c. of serum was inoculated at this time and again two hours later, when its temperature was 104° F. and the inoculated limb was very swollen and hot. By the 16th hour the temperature was 103° F., the sheep noticeably brighter, and the swelling of the limb felt cooler and less tense to the touch. Thirty-six hours after infection the animal appeared bright and normal except for its incapacitated leg, but by the eighth day only a slight limp remained and this eventually disappeared.

Had Sheep Nos. 2 and 9 not been pregnant they would in all probability have survived, as in both instances progressive local inoculation lesions had been arrested and recovery from these was occurring.

The experiments recorded in Table X are a testimony to the curative properties of immune serum and materially substantiate the claim that the inoculation of serum before or simultaneously with the contraction of infection is of prophylactic value.

Summary and Conclusions.

The great majority of cases of gas gangrene of sheep on the Romney Marsh are caused by *B. chauvœi*.

Parturient ewes suffer more than other sheep.

The disease is irregular in incidence and it is unlikely that active immunisation would be practised by owners, but prophylaxis by passive immunisation of individual ewes receiving assistance at lambing and thus exposed to infection would be undertaken. Accordingly experimental work has been concerned with passive immunisation. Experimental gas gangrene in sheep following upon intramuscular inoculation with cultures of *B. chauvœi* has been averted by the inoculation of hyperimmune serum prepared against ovine strains of *B. chauvœi* when the serum inoculated subcutaneously before, simultaneously with, or even four hours after the inoculation of culture, and by the intravenous inoculation of serum eight hours after infection. Further, when the inoculation disease has been allowed to progress until symptoms are acute and a fatal prognosis can be given from the symptoms shown, the disease may be overcome by the intravenous inoculation of large quantities of serum.

Preliminary experimental work was carried out upon guinea-pigs and mice, but final conclusions have been drawn by using sheep as the experimental animals.

These conclusions warrant the use of immune serum in the field on cases when there is reason to suspect that a *B. chauvœi* infection may be or may have been acquired.

COMPARISON OF REPEATED RAPID WHOLE-BLOOD, RAPID SERUM, AND TUBE AGGLUTINATION TESTS FOR THE DIAGNOSIS OF *S. PULLORUM* DISEASE.*

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In a previous paper[2] it was reported that there was a close agreement between the rapid whole-blood (stained antigen) and the rapid serum agglutination tests in the diagnosis of pullorum disease in 20 flocks (2,932 birds) tested once only. The purpose of the present paper is to report the results secured from repeated testing, for a period of three months at 30-day intervals, of a smaller group of birds by the rapid whole-blood (stained antigen), the rapid serum, and the tube agglutination tests.

Material and Methods.

A large flock of S.C.W., Leghorn, Light Sussex, R.I. Red and cross-bred fowls was tested by the rapid whole-blood agglutination test in December, 1931. From this flock 110 yearling females that had partially or completely recovered from the moult were taken at random for the experiment. Approximately two-thirds had reacted positively and the remainder negatively to the whole-blood agglutination test. The birds were confined in a 24-ft. by 16-ft. house, from which the peat moss litter was removed once after the first test was completed. Egg production for the three months was low (15 to 25 per cent.); one bird died between the second and third tests, two between the third and fourth tests, and one bird† was bled to death at the time the third test was made.

Whole-blood agglutination tests were made in the laying-house on January 10th, February 7th, March 5th and April 10th, and at the same time blood samples were taken. These were submitted later, in the laboratory, to the rapid serum and tube agglutination tests.

The technique of the whole-blood agglutination test was the same as that described in the previous paper.[2] The stained antigen used was obtained through the courtesy of Dr. M. Dorset, Bureau of Animal Industry, U.S. Department of Agriculture, Washington, D.C.

The technique of the rapid serum and tube agglutination tests was identical with that previously described by Biely.[1] The tube agglutination tests were conducted in dilutions of 1 : 25, 1 : 50 and

* Grant from the National Research Council of Canada.

† This bird reacted positively to the three agglutination tests. On post-mortem it had an enlarged liver and spleen typical of leukemia (myelogenous). The ovary showed typical lesions of pullorum disease, from which *S. pullorum* was isolated.

1 : 100. 0·4 c.c. of serum and 0·2 c.c. of antigen were used for the rapid serum tests. One lot of antigen for the tube agglutination tests and one lot of antigen for the rapid serum tests were used throughout the experiment.

Results.

Comparison of the Four Whole-blood Agglutination Tests.

A summary of the diagnoses of the repeated whole-blood agglutination tests (Table I) shows that 60 birds reacted consistently positive and 26 birds consistently negative to the four tests. Six birds which reacted negatively to the first test reacted positively in subsequent tests (Table III). Eighteen birds appeared to show one or more disagreements in the four tests since some were recorded as reacting sometimes positively and sometimes negatively. In six of the 18 birds the disagreements were apparently due to some fluctuations in the agglutination titre of the sera (Table II, birds 74, 159, 160, 210, 444 and 464). The disagreements in the remaining twelve birds, as will be seen from Table II, were obviously due to errors in technique or interpretation of reactions (Table II, birds 152, 186, 456, &c.). The total number of disagreements in the diagnoses, shown in the four tests of the 18 birds, was 24. As 435 diagnoses were made on the 100 birds in the four tests these disagreements constituted 5·5 per cent. of the total. The constancy of diagnoses in the four tests by the whole-blood agglutination test was therefore 94·5 per cent.

Comparison of the Four Rapid Serum Agglutination Tests.

Table I shows that the diagnoses of the four rapid serum agglutination tests were consistently positive in the case of 59 birds and consistently negative in 34. Eight birds which reacted negatively to the first test reacted positively in subsequent tests. In the case of nine birds there were disagreements in the diagnoses of the four tests. Some of these disagreements were apparently due to fluctuations in the agglutination titre of the sera (Table II, birds 74 and 464), and some to errors in technique (Table II, birds 11, 164, &c.). The total disagreement (11) in the diagnoses of the four tests by the rapid serum agglutination test was 2·5 per cent. of the 435 tests. The constancy of diagnoses in the four tests by the rapid serum agglutination test was therefore 97·5 per cent.

Comparison of the Four Tube Agglutination Tests.

Table I shows that 63 birds gave consistently positive reactions and 32 gave consistently negative reactions. Eight birds that gave negative reactions when first tested subsequently became positive reactors. Seven birds showed ten disagreements in the diagnoses of

the four tests. The total number of disagreements was 2·3 per cent. of the 435 tests. The constancy of diagnoses was therefore 97·7 per cent.

Comparison of Repeated Whole-blood, Rapid Serum, and Tube Agglutination Tests.

Table I shows that 57 birds reacted positively and 25 negatively to all four tests by the whole-blood, rapid serum, and tube agglutination tests. Eight birds that in the first test were negative to all three agglutination tests subsequently gave positive reactions. Six of these were consistently positive to all three agglutination tests, while two were consistently positive to the rapid serum and tube agglutination tests only (Table III).

Twenty-two birds showed disagreements in diagnoses in the four tests with one or more of the three agglutination tests. A summary of the diagnoses is shown in Table II. It will be seen that birds 152, 186, &c., which were recorded as reacting positively to the second test by the whole-blood agglutination test, reacted negatively to the first, third and fourth tests. These birds reacted consistently negative with the rapid serum and tube agglutination tests. Similarly, birds 11, 164, &c., which were recorded as reacting negatively in the first test with the rapid serum agglutination test, reacted positively to the first test by the whole-blood and tube agglutination tests, and to all subsequent tests by all three agglutination tests. Obviously the disagreements in the diagnoses of these birds were due to errors in technique or interpretation of reactions.

On the other hand, several of the 22 birds showed disagreements in the diagnoses of the four tests by all three agglutination tests. The disagreements in the diagnoses of these birds were apparently due to fluctuation in the agglutination titre of the sera. Thus birds 159 and 160 gave positive and negative reactions to the whole-blood test, positive and suspicious to the tube agglutination test, and negative and suspicious reactions to the rapid serum test; similarly, bird 444 was consistently positive to the tube agglutination test, positive and negative to the whole-blood test, and negative to the rapid serum test, giving one questionable reaction. Our data do not furnish a satisfactory explanation of these disagreements. However, since the number of birds which show such discrepancies is relatively small, they do not affect significantly the results of the retests.

Definite evidence has been obtained in this experiment to show that, in an infected flock, birds that have reacted negatively may react positively at subsequent tests when kept in contact with infected birds. Table III shows eight birds that were negative to the whole-blood, rapid serum and tube agglutination tests in January, and that reacted positively in later tests. One of these showed a positive reaction to all three agglutination tests in February. This bird and four others were positive to all three agglutination tests in March,

and three additional birds were positive in April. Of the 45 birds that were negative to all three agglutination tests in January, eight, or 17·7 per cent., reacted positively to all tests before the end of the experiment.

Several birds that reacted positively to the first and second tests reacted negatively to subsequent tests (Table II, birds 74 and 464). It is interesting to note that the change in reaction (from positive to negative) was recorded by the whole-blood, rapid serum and tube agglutination tests. While the results suggest that a decrease in agglutinins occurred, as shown by all three agglutination tests, the experiment was not carried out long enough to warrant definite conclusions.

Discussion.

The data obtained in this investigation show that a close agreement was secured in the diagnoses of the four tests by the whole-blood, rapid serum and tube agglutination tests. When the whole-blood agglutination test is compared with the rapid serum agglutination test it will be seen that there was agreement in 93·8 per cent. of the 435 diagnoses. This agrees closely with the percentage of agreement (98·7 per cent.) obtained in a previous investigation, in which 2,932 birds were tested by the rapid whole-blood and rapid serum agglutination tests once only.

When the whole-blood agglutination test is compared with the tube agglutination test, 93·6 per cent. of agreement is obtained in the 435 tests. This agrees closely with the percentage of agreement obtained by Bleecker[3] and by Bunyea and Hall.[4]

While the present investigation is not directly concerned with a comparison of the rapid serum and tube agglutination tests, it is interesting to note that 94·7 per cent. of agreement was obtained in the diagnoses by the tube agglutination tests.

The whole-blood, rapid serum and tube agglutination tests agree in 91·3 per cent. of the 435 diagnoses. Since each bird was tested four times by each of the three agglutination tests, there were actually 1,305 tests made. In these tests there were only 78 disagreements.

When the results of any one of the different agglutination tests used are compared for the four successive tests it will be seen that there was 94·5 per cent. agreement in the retests with the whole blood agglutination test, 97·5 per cent. agreement with the rapid serum agglutination test, and 97·7 per cent. agreement with the tube agglutination test.

The high percentage of agreement obtained in a comparison of the whole-blood, rapid serum and tube agglutination tests, and the constancy of diagnosis of the four tests by each agglutination test, shows that all three agglutination tests are reasonably comparable and constant in diagnosis.

Summary.

1. Four tests for pullorum disease were made at monthly intervals on 110 yearling fowls by the whole-blood, rapid serum and tube agglutination tests. 435 diagnoses were made with each agglutination test.

2. The percentage of agreement in diagnosis between the different agglutination tests was as follows : 91·3 per cent. by all three agglutination tests ; 93·8 per cent. by the whole-blood and rapid serum agglutination tests ; 94·7 per cent. by the rapid serum and tube agglutination tests ; and 93·6 per cent. by the whole-blood and tube agglutination tests.

3. The percentage of agreement of the four repeated tests by the same agglutination test was as follows : whole-blood agglutination test 94·5 per cent. ; rapid serum agglutination test 97·5 per cent. ; and tube agglutination test 97·7 per cent.

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[3]Bleecker, W. L. 1931. J. Am. Vet. Med. Assn., **78,** 518-526.

[4]Bunyea, H., and Hall, W. 1932. J. Am. Vet. Med. Assn., **80,** 491-496.

Table I.

Summary of Diagnoses of the Four Tests by the Whole Blood, Rapid Serum and Tube Agglutination Tests.*

	Number of Birds that Reacted.		*N/P* †	*Number of Birds that Showed Disagreement.*
	Consistently Positive.	*Consistently Negative.*		
Whole-blood	60	26	6	18
Rapid Serum	59	34	8	9
Tube	63	32	8	7
All three tests ...	57	25	6	22

*One bird was tested twice only, while three birds were tested three times only.

†N/P.—These birds reacted negatively to the first test and positively to subsequent tests (see Table III).

TABLE II.

SUMMARY OF AGGLUTINATION DIAGNOSES OF BIRDS SHOWING DISAGREEMENT IN ANY ONE OF THE FOUR TESTS WITH THE WHOLE BLOOD, RAPID SERUM AND TUBE AGGLUTINATION TESTS.

Number of Bird.	*Whole Blood Test No.*				*Rapid Serum Test No.*				*Tube Test No.*			
	1	2	3	4	1	2	3	4	1	2	3	4
11	P	P	P	P	—	P	P	P	P	P	P	P
30	S	S	S	—	—	—	—	—	—	—	—	—
55	—	P	?	—	—	—	—	—	—	—	P	—
74	P	P	?	—	P	P	P	—	P	P	S	—
152	—	P	—	—	—	—	—	—	—	—	—	—
153	—	P	P	—	—	P	P	P	—	S	P	P
159	P	P	—	P	—	S	—	—	P	P	S	P
160	P	P	—	—	—	S	—	—	P	S	P	?
164	P	P	P	P	—	P	P	P	P	P	P	P
186	—	P	—	—	—	—	—	—	—	—	—	—
210	—	P	—	—	—	S	—	—	—	P	P	—
329	—	—	P	—	—	—	P	P	—	—	P	P
444	P	P	—	—	—	?	—	—	P	P	P	P
445	—	P	—	—	—	—	—	—	—	—	—	—
455	—	—	P	—	—	—	—	—	—	—	—	—
456	—	P	—	—	—	—	—	—	—	—	—	—
464	P	P	—	—	P	P	—	—	P	P	P	—
467	P	P	P	P	—	P	P	P	P	P	P	P
478	P	P	P	—	P	P	P	P	P	P	P	P
479	—	—	—	—	—	—	—	—	—	S	S	S
481	—	P	—	—	—	—	—	—	—	—	—	—
497	P	P	—	P	P	P	P	P	P	P	P	P

— = Negative ; P = Positive ; S = Suspicious ; ? = Doubtful.

TABLE III.

SUMMARY OF DIAGNOSES OF BIRDS THAT REACTED NEGATIVE TO THE FIRST TEST AND POSITIVE TO SUBSEQUENT TESTS.

Number of Bird.	*Whole Blood Test No.*				*Rapid Serum Test No.*				*Tube Test No.*			
	1	2	3	4	1	2	3	4	1	2	3	4
29	—	—	—	P	—	—	—	P	—	—	—	P
153	—	P	P	—	—	P	P	P	—	S	P	P
167	—	—	P	P	—	—	P	P	—	—	P	P
329	—	—	P	—	—	—	P	P	—	—	P	P
468	—	—	P	P	—	—	P	P	—	—	P	P
470	—	—	P		—	—	P		—	—	P	
492	—	—	—	P	—	—	—	P	—	—	—	P
498	—	—	—	P	—	—	—	P	—	—	—	P

THE EFFECT OF SPIROCHETE AND TRYPANOSOME INFECTION ON THE DISTRIBUTION OF CHEMO-THERAPEUTICAL DRUGS.

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In previous researches* I have recorded the fact that it is impossible to effect a cure in mice infected with trypanosomes and recurrent spirochetes with doses of different drugs—germanin, trypaflavin, sulfureum, &c.—which suffice to bring about complete sterilisation in control animals infected only with trypanosomes. In searching for the cause of this phenomenon I was able to prove that spirochete infection exerts an unfavourable influence on the activity of the reticulo-endothelial cell system. I found that when on the culmination of the spirochete infection, or soon after it, I injected colloidal gold solution, the deposition of the gold grains in the Kupffer cells of the liver was to a large extent prevented, and no grains of gold could be detected in the perifollicular phagocytic cells of the spleen. I have concluded from this fact (1) that spirochete infection paralyses the phagocytic property of the reticulo-endothelial cells, and, at the same time, their immune biological functions ; and (2) I have also referred to the circumstance that the distribution of certain chemotherapeutical drugs, such as arsenobenzols, which, in normal animals, are deposited in the cells of the reticulo-endothelium, suffers great displacement in animals infected with recurrent spirochetes. To establish this I have examined the effect of the recurrent spirochete and trypanosome infection on the distribution of arsenobenzols in the body.

In these experiments I used white mice, which I infected partly with the European recurrent spirochete, partly with *Trypanosoma equiperdum*. At the height of the infection I injected the animals with four sorts of arsenobenzol, namely, neo-, silver, neo-silver and sulfoxysalvarsan. The mice were killed from 30 minutes to 24 hours after the injection because, according to Jancso, in normal animals the deposition of the arsenobenzol in the tissues is already completed in 30 minutes. For controls I regularly used animals which were treated with doses of arsenobenzol similar to those given to the infected animals. The parasite content of the blood was controlled microscopically. For the demonstration of the arsenobenzol I used the silver nitrate and glycerine method (Jancso). In order to observe the effect of trypanosome infection on the function of the reticulo-endothelial system colloid gold solution was injected intravenously, because neo-salvarsan shows a similar distribution in the

*Zbl. Bakter., Abt. 1, Bd. 123, pp. 140, 1931.

tissues to that of trypanblue ; for determining the finest differences I have in some cases given trypanblue intravenously.

In view of the object of my experiments, I first tried the effect of spirochete infection on the distribution of arsenobenzol.

Mouse No. 1.—Weight 20 gr. Injected intravenously with 0·7 c.c. of a 1 per cent. neosalvarsan solution. After the injection no clinical symptoms could be observed. The animal was killed after 30 minutes. Histological examination showed that in the capillaries of the liver and lung, the wall of the blood vessels in general, and the surrounding tissues as well as the trabeculæ of the spleen, the ammoniac-silver-nitrate was reduced to a brownish-black colour.

Mouse No. 2.—Weight 20 gr. At the height of the recurrent infection (parasite content of the blood ++++) 0·7 c.c. of 1 per cent. neosalvarsan solution was injected intravenously, and the animal was killed 30 minutes afterwards. After the injection the animal showed difficulty in breathing and refused to move.

Histological examination showed that the walls of the blood vessels of the lungs and liver, as well as the surrounding tissues, reduced the silver to a brownish-black colour, while the trabeculæ of the spleen did not show any reduction.

These experiments show that as a result of the infection with recurrent spirochetes the distribution of neosalvarsan was altered, inasmuch as 30 minutes after the injection none of it was deposited in the trabeculæ of the spleen. This phenomenon was obvious only on the culmination of the spirochete infection ; otherwise the deposition of the neosalvarsan in the spleen, on account of the infection, was present to a smaller extent.

I must here remark, it is observable in the organs of the control animals that neosalvarsan does not become deposited in the reticulo-endothelial system, but shows a high affinity for the connective tissues. Just on that account it seems the more interesting that the spirochete infection is able to check the deposition of neosalvarsan in the interstitial tissue of the spleen. Neosalvarsan, in its tendency to be deposited mostly in connective tissues, resembles several vital dyes, such as trypanblue ; consequently, it seems justifiable to assume that spirochete infection can change the distribution of vital dyes also. To test this supposition I injected trypanblue into mice infected with recurrent spirochetes, and at the same time into control animals.

Mouse No. 3.—Weight 20 gr. Injected intravenously with 0·5 c.c. of 1 per cent. trypanblue solution ; animal killed within an hour afterwards.

Histological examination showed that the Kupffer cells of the liver contained blue grains, equally distributed ; the trabeculæ of the spleen were stained blue.

Mouse No. 4.—Weight 20 gr. Injected intravenously with 0·5 c.c. of 1 per cent. trypanblue solution on the culmination (+ ++ +) of the spirochete infection, and killed 50 minutes afterwards. Difficulty in breathing had been observed after the injection. Macroscopic examination showed no differences as compared with the organs of the control animal.

Histological examination showed that only the Kupffer cells of the liver contained grains here and there, but the tissues surrounding the blood vessels were stained intense blue, contrary to what occurred in the controls. In the spleen the connective tissues were not so deeply stained as in the controls.

As can be seen from these experiments, in the body of an animal infected with recurrent spirochetes the deposition of the trypanblue, similarly to neosalvarsan, becomes altered, as the deposition in the parenchyma organs is checked by the infection. As my previous researches had indicated that the altered deposition of the colloidal gold caused by spirochete infection could be attributed mainly to the altered function of the reticulo-endothelial system, I thought that the distribution of these chemotherapeutical drugs would be changed first in those which become deposited in the reticulo-endothelial system—in opposition to the neo-salvarsan and vital dyes. To test this I have examined how the deposition of the silver- and neo-silver-salvarsan, which under normal conditions takes place in the reticulo-endothelial system, becomes changed on account of the spirochete infection.

Mouse No. 5.—Weight 20 gr. Injected intravenously with 0·3 c.c. of 1 per cent. silver-salvarsan solution and killed after 30 minutes. No serious symptoms were observed after the injection.

Histological examination showed that the tissues of the lungs were uniformly impregnated with arsenobenzol grains. The Kupffer cells of the liver, and also the phagocytic cells of the spleen, contained arsenobenzol grains (See Figs. 1 and 2).

Mouse No. 6.—Weight 20 gr. Injected intravenously with 0·3 c.c. of 1 per cent. silver-salvarsan solution at the height of the spirochete infection and killed after 30 minutes. *After the injection the mouse showed difficulty in breathing and did not move spontaneously.*

Histological examination showed in the lung coarse arsenobenzol grains which were uniformly distributed ; in the Kupffer cells of the liver phagocyted salvarsan grains were observed only at some places, and were unequally distributed in the substance of the liver. In the perifollicular cells of the spleen no traces of salvarsan could be found ; arsenobenzol grains were irregularly deposited throughout the substance of the spleen. (See Figs. 3 and 4.)

Mouse No. 7.—Weight 20 gr. Injected intravenously with 0·5 c.c. of 1 per cent. neosilversalvarsan and killed after 30 minutes. No serious symptoms observable after the injection.

Histological examination showed that the lung was filled with equally distributed arsenobenzol grains ; the Kupffer cells of the liver and the perifollicular cells of the spleen contained numerous phagocyted salvarsan grains.

Mouse No. 8.—At the time of the highest degree (+ + + +) of spirochete infection, injected intravenously with 0·5 c.c. of 1 per cent. neosilversalvarsan solution, and killed after 30 minutes. During the whole 30 minutes the animal did not move spontaneously, and it breathed with difficulty as compared with the control animal.

Histological examination showed that the lung contained coarse grains of arsenobenzol ; phagocyted grains could be seen at some places in the Kupffer cells of the liver, but no traces of phagocyted salvarsan could be found in the spleen.

When one considers the distribution of silver- and neosilver-salvarsan in the system of animals infected with recurrent spirochetes,

it is apparent that, compared with normal animals, it is disturbed, just as is the distribution of colloid gold. Thus the deposition of the first two mentioned drugs, which under normal condition takes place in a high degree in the Kupffer cells of the liver and perifollicular cells of the spleen, will be altered in animals infected with recurrent spirochetes. In these animals phagocyted salvarsan grains (Figs. 1, 2, 3 and 4) could be seen only in some places in the Kupffer cells, but none anywhere in the perifollicular cells of the spleen.

We can thus observe the changed distribution of the arsenobenzol in the tissues in the different degree of infection of animals infected with recurrent spirochetes (parasite content of the blood ++ or more). *The absence of phagocyted forms in the spleen can only be observed in the highest degree of infection.* This disorder of the function of the reticulo-endothelial system does not constantly remain, but disappears after the attack parallel with the disappearance of the spirochetes from the body; thus there was no difference in the distribution of the arsenobenzol on the day following the disappearance of the spirochetes from the blood.

In searching for an explanation of this phenomenon it appeared not improbable that the spirochetes changed the colloid-chemical structure of the blood in such a way that arsenobenzols are not precipitated in the same measure as in normal animals. In consequence of this, pure arsenobenzol grains would be formed and deposited in the organs. This possibly, however, could be excluded when one is following the accumulation of arsenobenzols in animals infected with recurrent spirochetes. Compared with the control animals, there are important differences only in the deposition in the phagocytic cells of the liver and spleen.

In animals infected with spirochetes a real difference cannot be observed in the content of arsenobenzol grains in the lung. Further, one sees that the arsenobenzol grains are scattered in the substance of the lung, but they do not get into the perifollicular cells. Examinations therefore show that *spirochete infection does not essentially influence the precipitation of the arsenobenzols.*

Taking all these points into account, one might think that the spirochetes produce substances which, passing into the blood, paralyse the activity of the phagocytic cells. To exclude this possibility I carried out experiments in which, before treating mice with silver-arsenobenzol, I injected them with 0·2 c.c. of centrifuged blood serum taken from animals in which there was a high degree (++++) of recurrent infection. In these experiments, however, I could not find any essential difference in the distribution of arsenobenzol in the animals treated with blood serum and the control animals. It may, therefore, be assumed that *spirochetes do not produce materials which, when they get into the blood stream, essentially influence the phagocytic ability of the reticulo-endothelial cells.*

Finally, one could come to no other conclusion than that the phagocytic ability of the reticulo-endothelial cells is disturbed because

they are filled with phagocyted spirochetes. I have examined this phenomenon on the ground of the supposition that, if filling the cells of the reticulum with albumen substances, such as the debris of spirochetes, causes these cells to lose their phagocytic ability, then, on the culmination of trypanosome infection, the phagocytic ability of the mesenchymal cells should be similarly hindered ; for it can be taken as certain that mesenchymal cells phagocytise trypanosomes just as they do spirochetes. I have therefore examined how the trypanosome infection influences the functions of the reticulo-endothelial cells. In the course of these examinations I have observed that there is no change in the distribution of colloid-gold, silver-, or neosilversalvarsan, even in the highest degree of trypanosome infection. It is thus obvious that the trypanosome infection does not in the least influence the phagocytic ability of the reticulo-endothelial cells. It follows that the deposition of albumen, in the form of debris of spirochetes or trypanosomes in the reticulo-endothelial cells, does not suffice to diminish the phagocytic ability of these cells. In trypanosome infections we see the same phenomenon that was observed on anodcolloids. As we know from the literature on the subject, it is impossible to fill the mesenchymal cells to such a degree with one kind of anodcolloid that they are not able to absorb other colloids. It may, therefore, be concluded that the deposition of colloid-gold and arsenobenzols in the reticulo-endothelial cells is not hindered simply by the fact that they are filled with spirochetes ; probably the albumen of the bodies of the spirochetes contains some kind of toxin which poisons the cells of the reticulo-endothelium in a way similar to colloid-copper solution. This supposition is confirmed partly by the observation that in chronic spirochete infections, of the fowl for instance, we generally find widespread necrosis in the parenchyma organs. To examine this question, I injected intravenously 0·003 gr. silversalvarsan into mice (weight 20 gr.) on the culmination of spirochete infection. For controls I used animals treated in the same way. The mice were killed after 30 minutes to 24 hours after the injection.

Histological examination showed that, while in control animals the deposition of the arsenobenzol was completed in about half an hour, in those infected with spirochetes there were no traces of arsenobenzol in the spleen, and only a small quantity of phagocyted grains in the liver. In further examinations I found that in the animals infected with recurrent spirochetes the silversalvarsan was deposited gradually.

In the highest degree of infection, two hours was the shortest time after the injection in which deposition was observable in the Kupffer cells to the same degree as in the controls. The deposition in the perifollicular cells of the spleen, however, was still disturbed.

Afterwards I injected intravenously 0·003 gr. colloid-gold into a 20 gr. mouse at the time of the highest degree of infection. When the animal was killed after two hours it was found that the Kupffer

cells of the liver presented an obviously hindered phagocytosis, while the phagocytosis of the perifollicular cells of the spleen was checked only to a certain degree.

It appears from these experiments that, at the culmination of recurrent infection the deposition of arsenobenzol, as well as of colloid-gold, is to some extent hindered. As a later period after the injection the distribution shows no essential difference as compared with the controls.

It must, therefore, be accepted as a proved fact that the *paralysing effect* exerted on the reticulo-endothelial system in recurrent infection *is transitory*. The phagocytic ability of the mesenchyma cells of the reticulo-endothelial system is hindered only on account of the recurrent infection. The deposition of the different substances differs only in the matter of time.

The change which occurs in the distribution of the arsenobenzols in recurrent infection can also be explained by the fact that in recovery from infection the spirochetes containing the arsenobenzols are deposited in the cells of the reticulo-endothelium.

Finally, I examined whether the spirochete infection does not essentially hinder the rapid selection of the arsenobenzols. I took for this purpose sulfoxysalvarsan, which, as is well known, shows great affinity for the kidneys. In these experiments I found that in animals infected with recurrent spirochetes and treated with 0·01 gr. sulfoxysalvarsan the kidneys presented the same degree of reduction of the silver-ammoniac as the kidneys of the control animals. It can, therefore, be stated that the rapid selection of the salvarsan was not influenced by the spirochete infection ; hence, comparing the results of these examinations with the data in literature, it is apparent that they can only be explained by the results of the experiments described above. For example, we find in them an acceptable explanation of the fact that Feldt and Heise did not obtain good results in the demonstration of salvarsan in tissues by the micro-method of Jancso. In his researches he generally used animals infected with spirochetes, in which, as my experiments have shown, there is a hindrance to the deposition of arsenobenzol ; on account of this, in the tissues of animals killed 30 minutes after the injection there are few or no demonstrable arsenobenzol grains.

My experiments are in accord with those of Schlossberger and Menk, who found that animals infected with recurrent spirochetes tolerate only smaller quantities of the chemotherapeutical drugs than control animals or animals infected with trypanosomes. The explanation of this is that when arsenobenzol is given intravenously the deposition of the precipitates after the injection is hindered in the case of animals infected with recurrent spirochetes. In these cases, therefore, favourable conditions are given for the development of arsenobenzol emboli.

My experiments also serve to explain the results obtained by Milbrandt, published recently. He showed that in animals in which

the liver had been injured by treatment with chloroform (parenchymal degeneration) salvarsan often causes shock symptoms. He therefore dissented from the opinion generally accepted that the acute shock symptoms are caused by arsenobenzol emboli, and emphasised the important rôle of the liver and sympathetic nervous system in the development of these symptoms.

A consideration of the results of my experiments with animals infected with recurrent spirochetes will show that, on account of the decrease of the function of the reticulo-endothelial cells, the animals develop more obvious acute toxic symptoms when given salvarsan either in well tolerated doses or in toxic amounts. This can be explained by the fact that precipitation of arsenobenzol does not occur in the parenchymatous organs. It is quite possible that by injuring the function of the liver with chloroform the distribution of arsenobenzol in the parenchyma organs can be altered. The fact that when the liver is injured in its functions arsenobenzol precipitates cannot be deposited, but are found in great quantities in the lungs, must be held responsible for the appearance of the acute symptoms. Chloroform poisoning has, therefore, the same result as injury of the function of the reticulo-endothelial apparatus with colloid copper or recurrent infection.

Thus, what is responsible for the sensitiveness to arsenobenzol of animals treated with chloroform is the fact that arsenobenzol cannot in such animals be deposited in the tissues.

Conclusions.

1. Recurrent infection highly influences the function of the Kupffer cells of the liver as well as that of the perifollicular cells of the spleen, and hinders the phagocytic ability of these cells at the culmination of infection.

2. Recurrent infection alters especially the deposition of those chemotherapeutical drugs which are regularly deposited in the reticulo-endothelial cells, but the distribution of drugs which have affinity for the connective tissues (*e.g.*, neo-salvarsan) is also disturbed by the spirochete infection.

In the white mouse, on the culmination of the recurrent infection 30 minutes after the injection, the deposition of arsenobenzol in the liver was highly checked, and in the spleen there was no phagocytosis of salvarsan grains.

Trypanosome infection did not essentially influence the distribution of colloid gold or of arsenobenzol. This infection does not influence the function of the mesenchymal system. This shows that saturation with phagocytosed parasites is not sufficient to account for the diminished function of the reticulo-endothelial system.

4. Recurrent infection paralyses the phagocytic ability of the reticulo-endothelial cells only for a short time, and both colloid-

gold and arsenobenzol are deposited two hours after the injection. The fact that the deposition of arsenobenzol in animals infected with recurrent spirochetes was belated can be explained by the possibility that these drugs are at first absorbed by the spirochetes themselves and become deposited only later with them.

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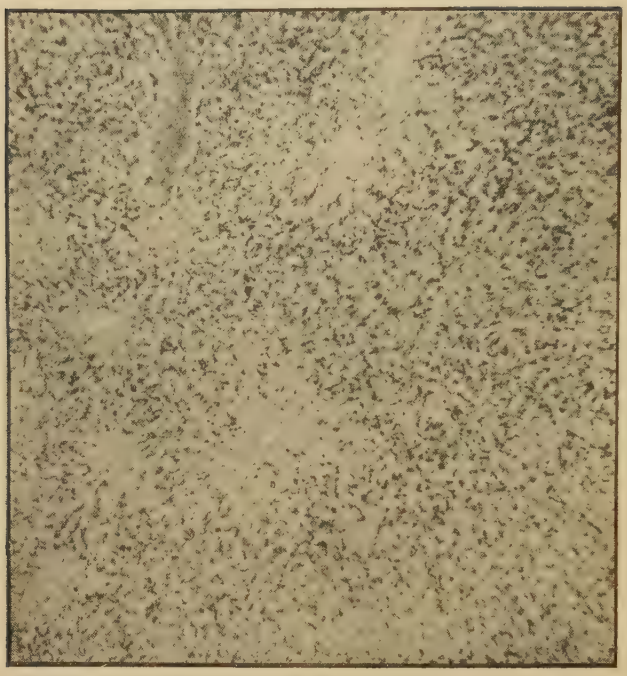

FIG. 1.
Distribution of silversalvarsan in the liver of control-animal. (30 minutes after the injection).

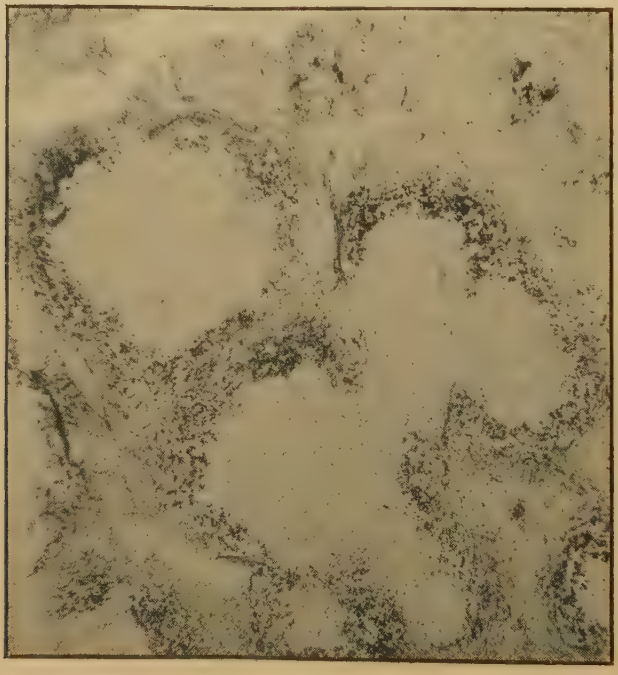

FIG. 2.
Distribution of silversalvarsan in the spleen of the control-animal. (30 minutes after the injection).

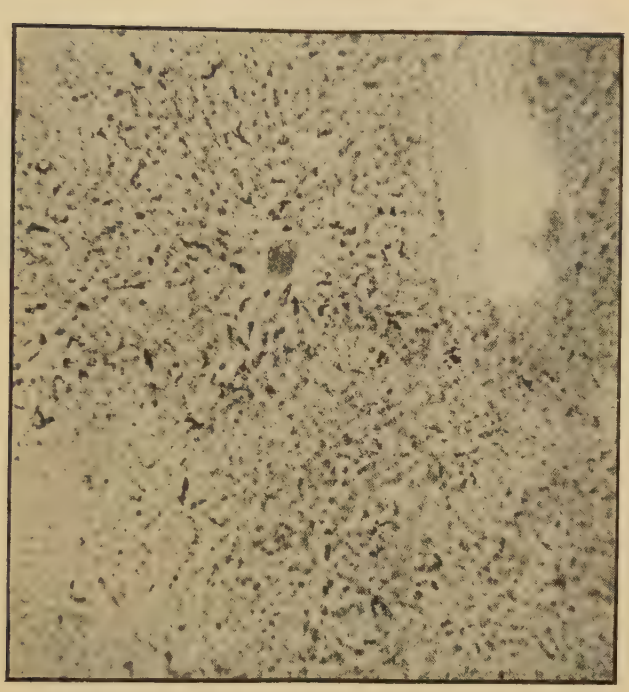

FIG. 3.
Distribution of silversalvarsan in the liver of mouse infected with recurrent spirochetes. (30 minutes after the injection).

FIG. 4.
Distribution of silversalvarsan in the spleen of mouse infected with recurrent spirochetes. (30 minutes after the injection).

STUDIES IN LOUPING-ILL

(An Encephalomyelitis of Sheep).

II.—TRANSMISSION BY THE SHEEP TICK, *Ixodes ricinus* L.

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From the Moredun Research Institute, Edinburgh.

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The sheep tick, *Ixodes ricinus* L., has long been suspected of being concerned in the transmission of louping-ill, and during the last 50 years many attempts have been made by different investigators to incriminate it as the vector of the causal organism. In no case, however, was it definitely proved that louping-ill had been produced, and the evidence obtained was often of a conflicting nature. Within recent years considerable advances have been made in the study of the causation of louping-ill, and a knowledge of the new facts has made an investigation of the transmission of this disease a comparatively simple matter.

Until the cause of louping-ill was definitely established (Pool, Brownlee and Wilson, 1930, and Greig, *et al.*, 1931), the transmission experiments of earlier investigators were necessarily inconclusive, and their results difficult to interpret. The most significant results were those of Stockman (1918-19), who produced febrile reactions by infesting sheep with ticks obtained from affected farms. It now appears, however (MacLeod, 1932, and Gordon *et al.*, 1932), that ticks on louping-ill affected farms may harbour the causal agent of a hitherto unrecognised disease of sheep, "tick-borne fever." It is probable that the reactions produced by Stockman were in some cases due to tick-borne fever, and his results therefore cannot be considered to afford definite experimental proof of the transmission of louping-ill by ticks.

In a previous paper (Gordon *et al.*, 1932) the following conclusions were reached as a result of experimental investigations of the authors and others: (1) Louping-ill is due to a filterable virus which is communicable to mice as well as to sheep. In the mouse, characteristic effects and lesions are produced by it. Thus the use of the mouse constitutes a valuable biological test for detecting the presence of this

virus. (2) By this means it has been shown that blood drawn during the febrile phase of louping-ill in sheep contains the virus, but the virus may be present in an infected sheep without producing typical louping-ill. (3) Such atypical affections may be manifested by a febrile reaction only, or they may be a cause of sudden death in sheep. (4) Recovery from the infection, whether naturally or experimentally produced, results in immunity of the sheep to intracerebral inoculation with the virus. (5) The investigation of louping-ill is complicated by the fact that a distinct disease, tick-borne fever, may coexist with it. Tick-borne fever is clinically and immunologically distinguishable from louping-ill. (6) Blood from a sheep infected with tick-borne fever is capable of causing a specific temperature reaction in normal sheep. After recovery, there is immunity to inoculation with infective material. Mice are not susceptible to this infection.

The above data afford means of determining whether a temperature reaction, which is not followed by symptoms of nervous disturbance, is or is not due to louping-ill infection. Firstly, if the thermal reaction is due to louping-ill infection the blood of the reacting sheep is infective for mice; secondly, the sheep after recovery is immune to intracerebral inoculation of virus.

In the following paper evidence of the transmission of louping-ill by the tick, *Ixodes ricinus* L., will be given, showing that: (1) the virus of louping-ill was recovered from ticks which had fed on affected sheep; (2) the presence of virus was demonstrated in the tissues of nymphs which as larvæ had fed on affected sheep; (3) louping-ill was transmitted to sheep by the bite of female and nymphal ticks which had respectively acquired virus as nymphs and larvæ; (4) tick-borne fever probably aggravates the course of the louping-ill attack.

Transmission by Emulsion of Gorged Ticks.

Although the lesions of louping-ill appear to be confined to the central nervous system, the virus can be demonstrated in the blood stream for a definite period during the course of the disease (Pool *et al.*, 1930, and Gordon *et al.*, 1932). It is, therefore, probable that ticks will acquire virus when ingesting the blood of an infected sheep. If the presence of virus can be demonstrated in ticks which have fed on infected hosts, it then remains to prove whether virus thus acquired will survive through a moult, or through the egg stage.

During the course of a field experiment (Gordon *et al.*, 1932) ticks were collected from sheep exposed to natural infection with louping-ill and tick-borne fever, and these ticks furnished material for the following experiments.

Experiment 1.

A sheep under observation in the field developed louping-ill and virus was shown to be present in its tissues. Female and nymphal ticks, at various stages of engorgement, were removed from the sheep at death and stored

for 24 days, when they were washed in saline and emulsified in a sterile mortar. Sufficient saline was added to make a 1 in 10 suspension, and this was centrifuged for 15 minutes at about 2,000 r.p.m. The supernatant fluid was inoculated into mice intracerebrally, as indicated in Table I.

TABLE I.

RESULT OF INOCULATION OF MICE WITH AN EMULSION OF TICKS WHICH HAD FED ON A CASE OF LOUPING-ILL.

Number of Mice Inoculated Intracerebrally.	*Suspension of Emulsified Ticks in Saline Dilution.*	*Result.*
2	1 in 100	0 0
4	1 in 10	0, +11 T.L.I., ++13T.L.I.
		2 Mice ++ 6 T.L.I.
		3 Mice +++ 6 T.L.I.

0 = "No take."
+x = Death x days after inoculation.
T.L.I. = Typical louping-ill.

Experiment 2.

Ticks at various stages of engorgement were collected from a sheep which had developed a febrile reaction while exposed to natural infection. Following the febrile reaction this sheep died, and, although it had not shown symptoms of louping-ill, the ticks which had been collected from it were tested for the presence of louping-ill virus in the ingested blood. Fifteen days after collection some of the ticks were emulsified in saline and centrifuged, and the supernatant fluid was inoculated into mice, as described in Table II.

TABLE II.

A RECORD OF THE INFECTIONS RESULTING FROM THE INOCULATION OF EMULSIFIED TICKS. FOR PASSAGE OF VIRUS A SALINE SUSPENSION OF THE BRAIN OF THE INFECTED ANIMAL WAS USED.

Number of Mice Inoculated Intracerebrally.	*Suspension of Emulsified Ticks in Saline Dilution.*	*Result.*
2	1 in 10,000	0 0
2	1 in 1,000	0 0
2	1 in 100	0 0
2	1 in 10	+ 9 T.L.I. + 18 T.L.I.
		3 Mice +++ 6 T.L.I.
		2 Mice ++ 5 T.L.I.
		1 Sheep + 5 T.L.I.
		2 Mice ++ 6 T.L.I.
		2 Mice ++ 5 T.L.I.
		1 Sheep + 5T.L.I.
2 Mice 1 in 10		++6 T.L.I.
2 Mice 1 in 100		++6 T.L.I.
2 Mice 1 in 1,000		++6 T.L.I.
2 Mice 1 in 10,000		+6+7 T.L.I.
2 Mice 1 in 100		+8+9 T.L.I.
2 Mice 1 in 1,000		++9 T.L.I.
2 Mice 1 in 10,000		++14 T.L.I.
2 Mice 1 in 100,000		0 0

Abbreviations as in Table I.

Inference from Experiments 1 *and* 2.

The foregoing experiments show that when ticks are fed on sheep infected with louping-ill virus the virus may survive in the body of the tick for a period of at least 24 days. It now remained to decide whether virus thus acquired can survive through a moult of the tick and so be demonstrated in the succeeding stage, and also whether an infected adult tick transmits the virus to its progeny.

Emulsion of Larvæ from Females Exposed to Infection.

Experiment 3.

Of the ticks collected from the sheep referred to in the previous experiment and shown to have ingested virus, one fully gorged female was set aside. The larvæ bred from this female were emulsified in saline, centrifuged, and inoculated into three mice. One died of the operation ; two remained healthy for 21 days, when a negative result was accepted.

Transmission by Emulsions of Ticks after Moulting.

(*a*) *Emulsion of Nymphs Bred from Larvæ Exposed to Infection.*

Experiment 4.

Larvæ bred from females collected from healthy sheep, that is, " clean " larvæ, were infested on an experimental case of louping-ill (Ram 5) and fed during the course of the temperature reaction preceding its death from louping-ill. The fully gorged larvæ were collected and bred to the nymphal stage.

Some of these nymphs were emulsified in saline and centrifuged, and the supernatant fluid was inoculated into mice, as described in Table III.

TABLE III.

A Record of the Infections Resulting from the Inoculation of Emulsified Nymphal Ticks which had fed as Larvæ on an Infected Sheep.

Number of Mice Inoculated Intracerebrally.	*Suspension of Nymphal Ticks in Saline Dilution.*	*Result.*
3	1 in 20	+6 T.L.I. +8 T.L.I. +7 T.L.I.

6 Mice	+5 T.L.I. +5 T.L.I. +6 T.L.I. +6 T.L.I. +6 T.L.I. +6 T.L.I.	
2 Mice		+6 T.L.I. +6 T.L.I.

Abbreviations as in Table I.

(*b*) *Emulsion of Adults Bred from Nymphs Exposed to Infection.*

Experiment 5.

A sheep under close observation in the field died of louping-ill and virus was recovered from its tissues. During the course of the temperature reaction which preceded death three gorged nymphs were collected. These moulted to adult ticks, yielding two females and one male. An emulsion of these ticks was inoculated into two mice, neither of which developed louping-ill.

Inference from Experiments 4 *and* 5.

It appears that, in the case of the larvæ at least, louping-ill virus ingested by this stage can survive a moult, and can be demonstrated in the bodies of the resulting nymphs. In view of the small number of experiments it cannot be concluded from Nos. 3 and 5 that a negative result would always be obtained under these conditions.

It remained to prove whether ticks thus infected could transmit the virus to sheep by biting.

Transmission by Infestation with Ticks.

In the following experiments ticks were allowed to acquire infection by feeding on experimentally produced cases of louping-ill. These ticks were then bred to the next stage, and allowed to feed on normal sheep.

The technique employed was as follows: The scrotum of the ram was clipped and washed. The unfed ticks were placed in a cloth bag which was tied over the scrotum. The bag was removed after 24 hours, by which time the majority of the ticks had become attached. When the engorgement period was nearly over the bag was replaced and the gorged ticks removed from it daily.

Experiment 6, *Ram* 245.

This sheep was infested with nymphal ticks two months old, bred from larvæ gorged on Ram 5, an experimental case of louping-ill. The temperature of Ram 245 was recorded daily during the experiment. This animal did not exhibit any constitutional disturbance and its temperature remained normal for a period of fourteen days, when a negative result was accepted. This result was unexpected, as ticks from the same lot produced louping-ill when inoculated into mice (*vide* Experiment 4).

Experiment 7, *Ram* 285.

This ram was infested on August 22nd with female ticks bred from nymphs which had fed on Ram 7, an experimental case of louping-ill. Four females completed engorgement. The temperature of Ram 285 was recorded daily (Chart I). It will be seen that a temperature reaction commenced on the third day after infestation. The curve was diphasic in character and the reaction lasted for eleven days. On August 28th and September 3rd, blood drawn from this sheep was inoculated intracerebrally into mice. The mice remained healthy, but the result could not be taken as negative evidence of the presence of virus in the blood, since we now know that the most suitable dates for recovery of virus from the blood of this sheep would have been August 25th, 26th and 27th (Gordon *et al.*, 1932). In order to decide the cause of the temperature reaction, blood was again drawn from this sheep on September 4th, and inoculated subcutaneously into a normal sheep and a sheep immune to tick-borne fever. Neither of these sheep reacted. Seventeen days after its return to normal, Ram 285 was tested for immunity to tick-borne fever by the injection of virulent blood, and on the fourth day after it developed a temperature reaction which lasted for nine days. After recovery from this reaction the ram was tested for immunity to louping-ill by intracerebral inoculation of virus and did not react, whereas two control sheep inoculated at the same time developed typical louping-ill and were destroyed on the sixth day.

Accordingly, although the virus of louping-ill was not recovered from the blood of this sheep, the temperature reaction which followed infestation was probably due to louping-ill infection, since it was proved that the reaction was not due to infection with tick-borne fever, and since the sheep had acquired immunity to louping-ill.

In view of the above two results, the possibility had to be considered that the ticks used were infective, but were not capable of setting up a severe infection unless the resistance of the sheep was weakened in some way, such as by loss of bodily condition or by the presence of another disease. Under natural circumstances, tick-borne fever occurs along with louping-ill and is transmitted by the female and nymphal stages of the tick. Subsequent infestation experiments were therefore carried out in which tick-borne fever was induced in the sheep subsequent to, preceding, or simultaneously with, their exposure to biological infection with louping-ill, *i.e.*, to infection as a result of tick bite.

Infestation both with Louping-ill Infected Ticks and Tick-Borne Fever Infected Ticks.

(*a*) *Louping-ill followed by Tick-borne Fever.*

Experiment 8, *Ram* 245.

This ram was the animal recorded in Experiment 6. It failed to react after infestation with nymphs which as larvæ had ingested blood from a sheep harbouring louping-ill virus (*vide* Experiment 6). Fourteen days later it was infested with nymphs known to harbour the infective agent of tick-borne fever. A typical tick-borne fever reaction commenced on the fifth day and lasted over 15 days (Chart II). On the sixth day, when the temperature was 107·2° F., blood was drawn and inoculated into four mice without producing louping-ill. Blood was also inoculated into a normal sheep and a sheep immune to tick-borne fever. The normal sheep reacted and the immune sheep did not. Ram 245 was subsequently tested for immunity to louping-ill and died in six days after a typical attack of the disease. In this case, therefore, a tick-borne fever infection did not activate louping-ill virus which presumably had been introduced at the first infestation with ticks.

(*b*) *Tick-borne Fever followed by Louping-ill.*

Experiment 9, *Ram* 277.

Ram 277 was infested with females and larvæ bred from nymphs and females which had gorged on a case of tick-borne fever in the field, and typical tick-borne fever developed eight days later. On each of the first six days of the reaction blood from this ram was tested for the presence of louping-ill virus by inoculation into mice, and all the mice remained healthy.

On the ninth day of the reaction, when the temperature was 107·6° F., this sheep was infested with some of the nymphs infected with louping-ill as larvæ by feeding on Ram 5. (Some of the same group of nymphs were shown to be infected when emulsified and inoculated into mice (*vide* Experiment 4), while others had failed to produce a reaction when allowed to feed on Ram 245, Experiment 6). Ram 277 had a sustained high temperature for the four days following this second infestation (Chart III). On the fourth day, blood was drawn and inoculated into two mice, which developed

typical louping-ill on the fifth and seventh day respectively after inoculation. To confirm the diagnosis, virus was recovered from these mice and passed in series to other mice in which characteristic louping-ill developed.

On the eighth day after the second infestation the sheep was weak and lay most of the time. On the following day it was unable to stand ; breathing was laboured, but there were no characteristic nervous symptoms of louping-ill. In the evening the sheep died, and post-mortem examination revealed no gross lesions which might account for death, the organs being apparently normal. Saline emulsions prepared from the brain and spinal cord were inoculated into mice and the virus of louping-ill was recovered from both organs.

(c) Simultaneous Louping-ill and Tick-borne Fever.

Experiment 10, *Ram* 248

Ram 248 was infested with females and larvæ which in their previous stage had fed on a case of tick-borne fever in the field. At the same time it was infested with some of the Ram 5 strain of nymphs infected with louping-ill as larvæ. Chart IV depicts the temperature reaction following infestation. During the second febrile phase the sheep was dull and almost continuously lay apart from its fellows. On the fifteenth day after infestation the sheep was observed to be trembling violently ; it walked with difficulty, and occasionally showed twitching of the lips. The following day the animal was recumbent and exhibited generalised tremor of the superficial muscles. When raised to its feet it could stand only for a few seconds. On the sixteenth day the sheep was unable to rise, the hind quarters being paralysed, and at this stage it was killed by bleeding. Blood was drawn during the first febrile phase and inoculated into two mice, both of which developed louping-ill on the seventh day. After the death of the sheep louping-ill virus was detected in the brain and spinal cord by inoculation of saline emulsions of these tissues into mice. Towards the end of the second febrile phase blood was drawn and inoculated into one normal sheep and one immune to tick-borne fever. The normal sheep developed a tick-borne fever reaction, while the immune did not react. This was accepted as evidence that tick-borne fever infection was present in Sheep 248.

Experiment 11, *Ram* 283.

During the field experiment already referred to, a sheep exposed on pastures where louping-ill was prevalent developed a typical tick-borne fever reaction. At the end of the reaction the sheep developed symptoms of louping-ill and was killed. Presumably, then, towards the end of the thermal reaction the sheep was suffering both from tick-borne fever and louping-ill.

Gorged ticks collected from this sheep during the febrile phase were bred to the next stage and yielded numerous larvæ and eleven females. These were placed on Ram 283. Five females and numerous larvæ gorged. On the second day after infestation the temperature of this sheep rose to 106·4° F., and it was 107° F. on the following day. It then fell to 105·6° F., and remained low for another day, when it rose to 106·2° F. For the following six days its temperature was above 105·8° F., being usually over 106° F. It then subsided and subsequently remained normal. The sheep showed no clinical symptoms of louping-ill apart from a slight dullness and definite loss in bodily condition. During the course of the first temperature reaction blood was drawn and inoculated into two mice. These developed louping-ill on the sixth and seventh days respectively. During the second febrile phase blood was drawn and inoculated into one normal sheep and one immune

to tick-borne fever. The normal sheep developed a tick-borne fever reaction, while the immune sheep did not react, and this was accepted as evidence of tick-borne fever infection in Ram 283. After recovery this ram was inoculated subcutaneously with 5 c.c. of virulent blood from a case of tick-borne fever and did not react, whereas a normal sheep inoculated with a similar quantity of the same blood reacted severely. The ram was later tested for immunity to louping-ill by intracerebral inoculation of virus, and did not develop a reaction during the following eight days ; but on the ninth day it died of pneumonia. Two controls inoculated with the same material developed typical louping-ill and were destroyed on the sixth day.

Summary.

In three infestation experiments in which tick-borne fever was induced prior to, or simultaneously with, exposure to louping-ill infection, louping-ill developed in the experimental sheep. One of the sheep developed characteristic symptoms of the disease and died ; one died without having shown typical symptoms ; and one developed a thermal reaction but recovered. All three sheep were proved to have been infected with louping-ill virus, because (1) the specific virus was recovered from the blood during the febrile reaction, (2) the specific virus was recovered from the central nervous system of the two sheep which died, and (3) the sheep which recovered had acquired immunity to this virus.

Infestation with Ticks of Different Ages.

The results of the above five experiments suggested that sheep infected with tick-borne fever were more susceptible to infection with louping-ill by tick bite than were normal sheep. As an alternative explanation of the failure of the ticks to produce a louping-ill reaction in a normal sheep (Experiment 6), it might be suggested that for some time after the tick has moulted it is not capable of causing infection, *i.e.*, that there is an incubation period during which the virus is either not infective or has not reached the salivary glands of the tick. In order to test these two hypotheses, a further experiment was carried out. The material for this experiment was prepared as follows :—

A ram (No. 289) was inoculated intracerebrally with louping-ill virus and infested with larvæ on the following day. These larvæ were the progeny of gorged females collected from apparently healthy sheep in the field. The larvæ completed engorgement towards the end of the temperature reaction preceding death of the sheep, and they were collected and bred to nymphs. Another ram (No. 290) was inoculated subcutaneously with louping-ill virus, and four days later was infested with larvæ of similar history to those put on Ram 289. Gorged larvæ were recovered towards the end of the temperature reaction in the host and were bred to nymphs.

Experiment 12. *Rams* 284, 428, 429, 427 *and* 247.

The nymphs bred from the larvæ gorged on Rams 289 and 290 were used in three lots, each lot being of different age after moulting,

viz., (1) twelve days old, (2) 15 to 30 days old, and (3) 40 to 50 days old. Five rams obtained from a non-louping-ill farm were used for the experiment. For seven days before the sheep were infested their temperatures were recorded daily and found to be normal. The twelve day old ticks were placed on one ram, which was then inoculated subcutaneously with 5·0 c.c. of virulent tick-borne fever blood. The 15 to 30 day old ticks were placed on two rams, one of which was also inoculated with virulent tick-borne fever blood. The 40 to 50 day old ticks were placed on two rams, and one of these was also inoculated with virulent tick-borne fever blood. From this experiment it was hoped to determine (*a*) if there was an incubation period after moulting before the tick became infective, and (*b*) if the presence of tick-borne fever infection precipitated or aggravated a louping-ill infection.

Ram 284. *Infested with Ticks under Twelve Days old, and Inoculated Simultaneously with Virulent Tick-borne Fever Blood.*

Four gorged nymphs were recovered. The temperature rose on the fourth day after infestation, and on the sixth day was 107·4° F. It dropped on the eighth day to 105·2° F., then rose the following day to 107° F. On this day the sheep was observed to be dull. During the following two days the temperature dropped from 106° F. to 105·4° F., when the sheep was definitely dull. It lay down when being penned in the morning. At 4 p.m. it was in a state of generalised muscular tremor; it could still maintain stance when assisted to its feet, but staggered to the right, and was inclined to collapse to that side. It was then destroyed by bleeding. Throughout the course of its temperature reaction blood was drawn daily and inoculated into mice. Virus was demonstrated in the blood stream on the fourth, fifth, sixth, seventh, eighth and ninth day after infestation. After the sheep was destroyed virus was detected in the central nervous system by inoculation of saline emulsions of this tissue into mice.

Ram 428. *Infested with Ticks* 15 *to* 30 *Days old, and Inoculated Simultaneously with Virulent Tick-borne Fever Blood.*

Only two nymphs attached. The temperature of this sheep rose to 105·4° F., on the fourth day after infestation, and was 108° F. on the seventh day. For the next six days it remained over 106° F. During this period the sheep showed evidence of discomfort; breathing was hurried and there was a definite loss in physical condition. The temperature declined during the fourteenth and fifteenth days to 104° F., rose to 105·6° F. on the sixteenth day, and then dropped to 103·2° F. The following day the temperature was 102·4° F., and the sheep was unable to rise, breathing was rapid, and there was occasional violent kicking with the hind limbs. Intelligence was normal and the eye bright. It was destroyed by bleeding. Virus was recovered from the blood on the fourth day, and, after death, from the brain and cord.

Ram 429. *Infested with Ticks* 15 *to* 30 *Days old.*

Seven gorged nymphs were recovered. This ram gave a prolonged and irregular temperature reaction from the fifth to the twenty-sixth day after infestation. The temperature did not exceed 106° F., except on the eighth day, when it was 106·2° F. Virus was present in the blood on the sixth

and seventh day after infestation. Forty-one days after infestation this sheep was tested for immunity by intracerebral inoculation of louping-ill virus and remained normal.

Ram 427. *Infested with Ticks* 40 *to* 50 *Days old, and Inoculated Simultaneously with Virulent Tick-borne Fever Blood.*

Nine gorged nymphs were recovered. This ram developed a thermal reaction on the third day after infestation and inoculation, and its temperature was 107° F. on the eighth day. The following day, the temperature dropped to 104·8° F. The sheep was excited, and when walking showed excessive flexion of the hind limbs. Occasional swaying was apparent in its gait. The following day the inco-ordination of gait was more exaggerated ; the animal trembled on being handled, and it was seized with a violent convulsion when its temperature was being taken. The symptoms persisted for the following two days, after which it made a gradual recovery. Virus was present in the blood on the third, fourth, fifth and sixth days after infestation. Forty-one days after infestation, the ram was inoculated intracerebrally with louping-ill virus and remained normal.

Ram 247. *Infested with Ticks* 40 *to* 50 *Days old.*

Sixteen gorged nymphs were recovered. Chart V shows the reaction of this sheep, which died as a result of infection with louping-ill alone. On the fourth day the animal was observed to be dull ; during the following two days it was distinctly dull and stumbled occasionally when walking. It was inclined to lie apart from its fellows and did not feed. There was a marked loss in condition. On the seventh day, when the second febrile phase began, a fine tremor of the limbs was apparent ; the ram was disinclined to move and stood with drooping ears and nodding head or lay apart. There was slight salivation. The following day the temperature dropped to 102·4° F. The animal was very dull and almost unable to stand ; it refused to be driven and when left alone lay down. Later it was unable to stand. Death occurred on this day. Virus was present in the blood on the third, fourth, fifth, sixth and seventh day. After death, virus was recovered from the spleen, but not from the brain or cord.

From these results it appears that nymphs infected with louping-ill as larvæ can produce the disease as soon after moulting as they are capable of attaching themselves to sheep, that is, there does not appear to be an incubation period after the moult during which the tick is incapable of causing infection. Further, it is clear that in the transmission of louping-ill tick-borne fever is not a necessary factor, although the latter disease, by reducing the sheep's vitality, may predispose it to develop the typical nervous symptoms of louping-ill.

Discussion.

The occurrence of the tick, *Ixodes ricinus*, on the rough hill pastures of Scotland and its association with louping-ill has long been recognised. We can find no authenticated record of the occurrence of louping-ill on farms which are free from ticks, while on badly infected louping-ill farms ticks are very prevalent. Further, it is known that the louping-ill season corresponds with the period of activity of the ticks in nature. Scientific proof, however, of the transmission of the disease by the tick had hitherto been lacking.

The investigation of the biological transmission of louping-ill has been complicated by the fact that under natural conditions ticks on "diseased" farms harbour and transmit two infective agents, the virus of louping-ill and the infective agent of tick-borne fever.

In the experiments described in the preceding pages it has been shown that when ticks engorge on a sheep affected with louping-ill during the period of thermal reaction virus can be recovered from these ticks. Further, the virus thus acquired by larvæ is present in the succeeding nymphal stage.

A number of experiments are recorded here in which ticks infected with the virus in one stage were allowed in their next stage to feed on healthy sheep. In these infestation experiments the following were taken as criteria of louping-ill infection :—

(*a*) The sheep develops a febrile reaction, which may or may not be followed by symptoms of nervous disturbance and by death.

(*b*) The blood of a reacting sheep, drawn at the acme of the thermal reaction and inoculated intracerebrally into mice, produces symptoms of louping-ill followed by death.

(*c*) A sheep which has recovered from a febrile reaction caused by louping-ill infection is subsequently immune to the virus inoculated intracerebrally.

The failure of the first attempt to transmit the disease to a healthy sheep by tick bite led us to look for a predisposing factor, and it was found that sheep affected with tick-borne fever readily became infected with louping-ill when bitten by infected ticks. Later experiments, however, showed that tick-borne fever is not a necessary factor, since healthy sheep may acquire louping-ill as a result of the bite of infected ticks.

Nine cases of louping-ill were produced in sheep by infestation with ticks infected with louping-ill virus.

In six of these nine, the sheep were infected with tick-borne fever, either before, or simultaneously with, exposure to louping-ill infection. Three of the six exhibited the classical symptoms of louping-ill and died or were destroyed when moribund ; one died after exhibiting atypical symptoms, one exhibited symptoms of nervous derangement but recovered, and one recovered after undergoing a thermal reaction only. Louping-ill virus was recovered from the blood of all six sheep ; it was present in the central nervous system of the four which died, and the two which survived were subsequently immune to the disease.

Three cases of louping-ill were produced in which tick-borne fever was not a concurrent infection. One of the three sheep exhibited atypical symptoms and died ; virus was recovered from its blood and spleen. Two recovered after developing a thermal reaction, and were subsequently immune to the disease. Virus was recovered from the blood of one of these during the course of the reaction.

It appears from these results that infection of the central nervous

system occurred only in those sheep which were undergoing a concurrent infection of tick-borne fever, and it is possible that the presence of this disease facilitates the invasion of the central nervous system by the louping-ill virus. It has been shown, however, that sheep can become infected with, and even die from, louping-ill infection by tick bite, although tick-borne fever be not present.

There is little doubt, in the light of these results, that the tick, *Ixodes ricinus* L., is the common vector of louping-ill in nature, and it is probable that, in at least the majority of infections, tick-borne fever plays an accessory part in establishing the disease.

The type of reaction in a sheep infected by tick bite appears to be as follows : There is an incubation period of usually one to two days, after which the temperature rises. The maximum temperature is reached about the second day of the reaction, and then the temperature declines during the following two or three days. Death may occur at this stage, or there may be a second rise and fall of temperature. This second febrile phase is accompanied by the appearance of the first symptoms of nervous derangement, if these develop. Recovery, or an increase in the severity of the symptoms and ultimate death, may follow.

Ticks were found to be capable of producing louping-ill within twelve days after moulting. Since ticks, after moulting, require about eight or nine days to harden before they will attach themselves to a host, it follows that they are infective as soon as they are able to attach themselves.

It is well established that there is considerable difficulty in producing louping-ill experimentally unless the infective material be introduced directly into the central nervous system. It is, therefore, a striking fact that the tick by inoculating the virus into the peripheral blood stream can cause infection of the central nervous system. This difference in the results of biological and artificial inoculation, and the possible rôle played by tick-borne fever in facilitating infection by the biological method of inoculation, raises interesting possibilities, and is at present the subject of further study.

The discovery that louping-ill can be transmitted by the tick is of interest in view of the fact that the causal agent of this disease is a virus which exhibits neurotropic characteristics. Until the work of Montgomery (1917), confirmed by Daubney and Hudson (1931), there was no record of ticks transmitting a virus disease. These workers reported the transmission of the virus of Nairobi Sheep Disease by a tick, *Rhipicephalus appendiculatus*, and that is the only other known instance of ticks transmitting an ultramicroscopic virus, but in its case the virus is not neurotropic.

Conclusions.

(1) Louping-ill has been transmitted to sheep by the bite of nymphal and adult female *Ixodes ricinus* ticks which in their previous stage had engorged on sheep affected with the disease.

(2) The tick is infective as soon after moulting as it is capable of attaching itself to a host.

(3) Evidence suggests that tick-borne fever may have an important influence on the course of the disease. In the absence of tick-borne fever the typical nervous symptoms of louping-ill are unlikely to develop, although infection with the virus of the latter occurs.

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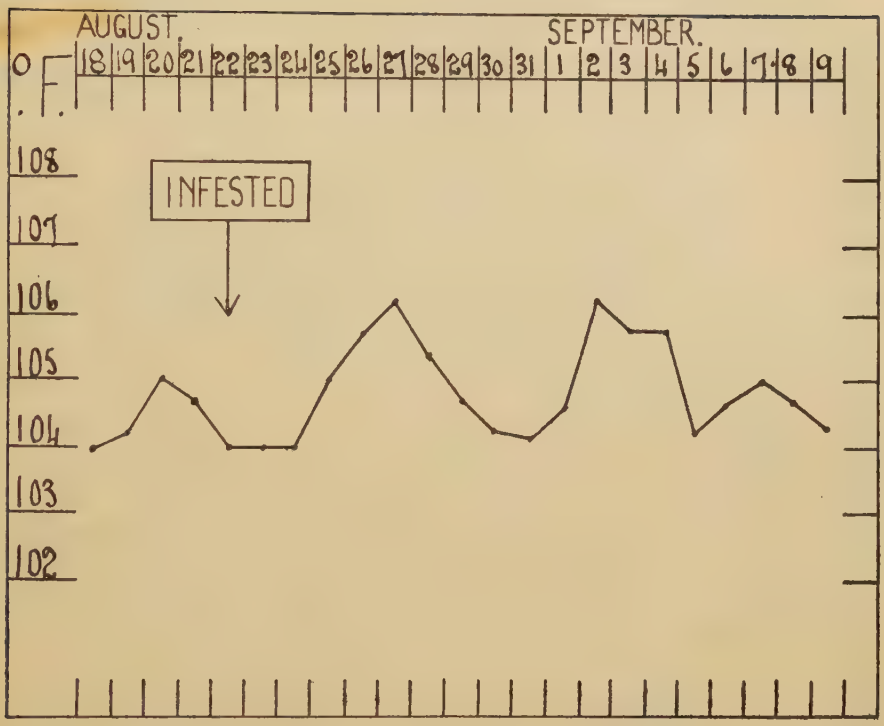
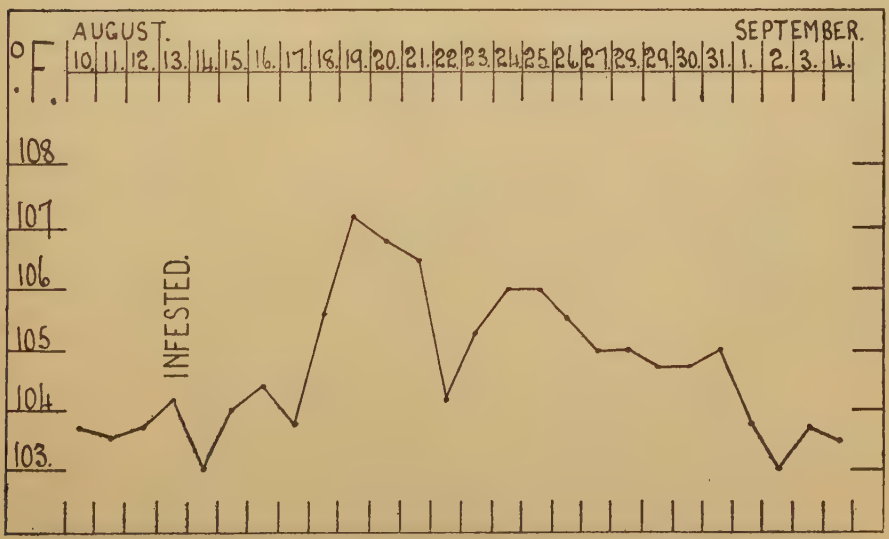
FIG 1.



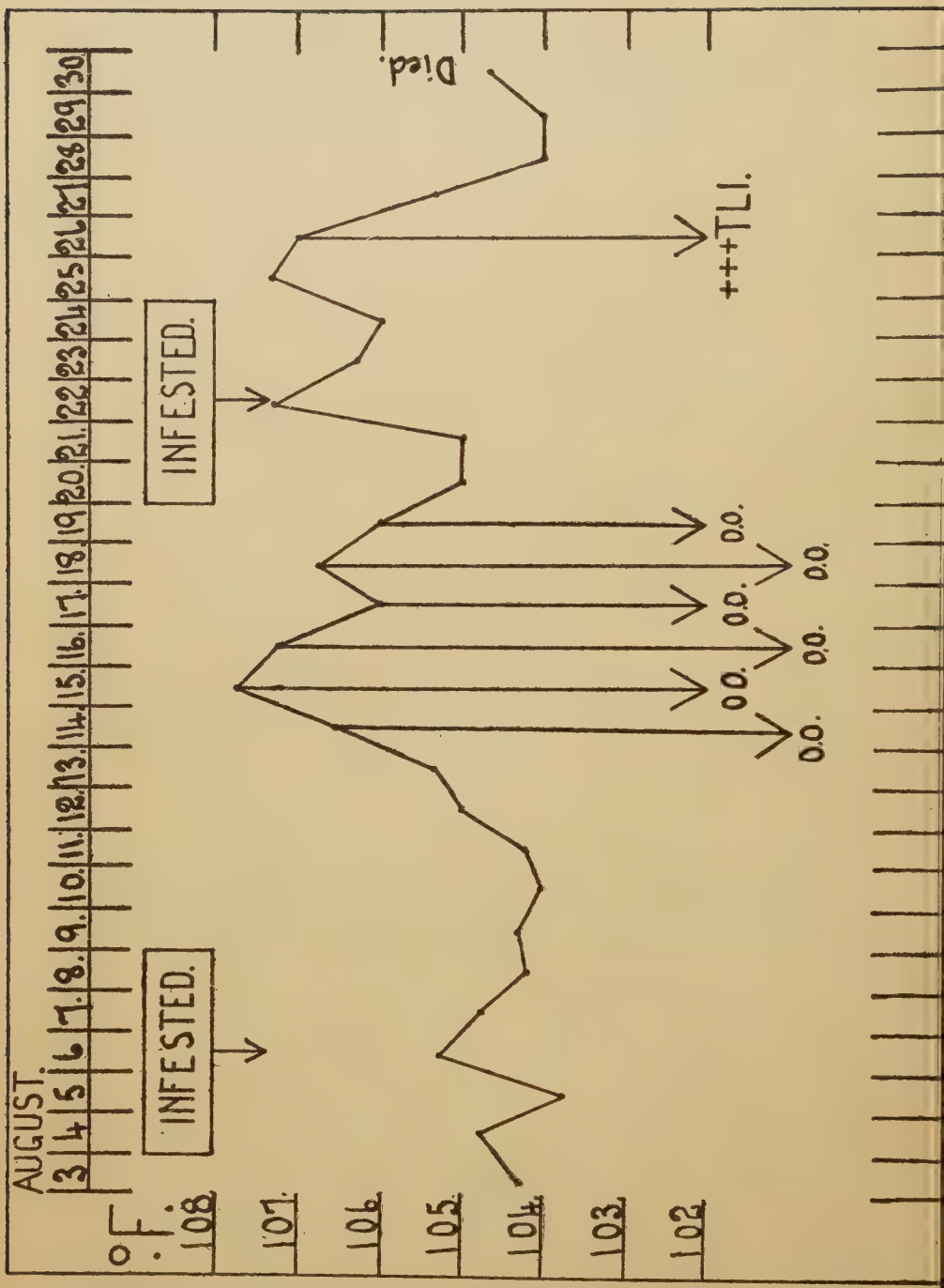
Sheep 285.—Temperature reaction following infestation with louping-ill infected ticks.

FIG 2.



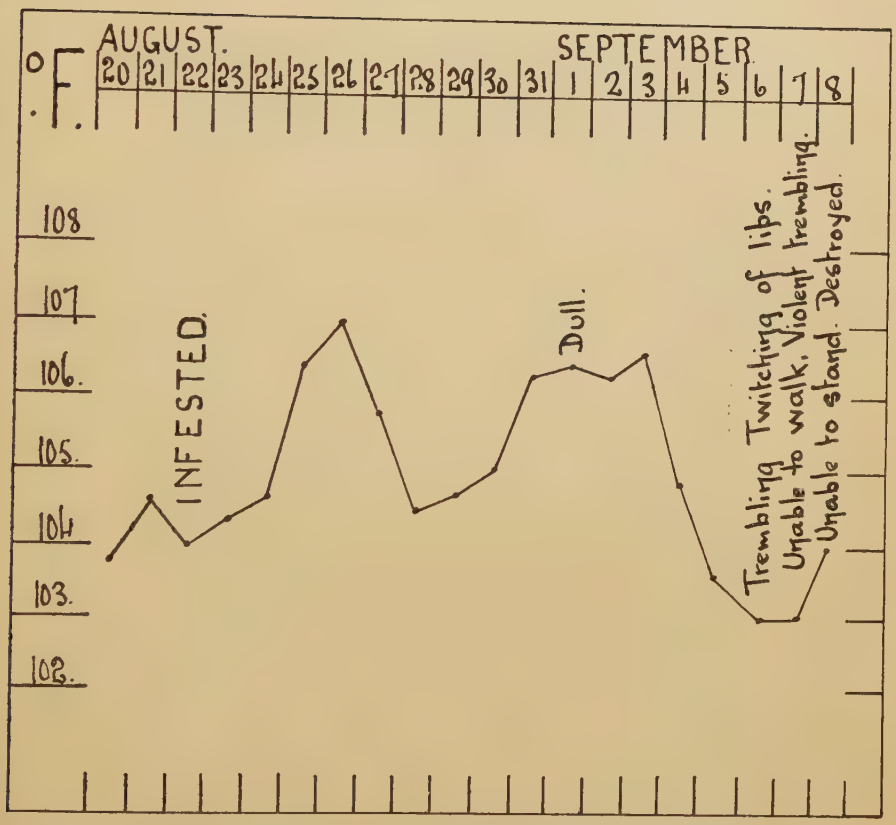
Sheep 245.—Typical tick-borne fever reaction, following infestation with infected nymphs.

FIG. 3.



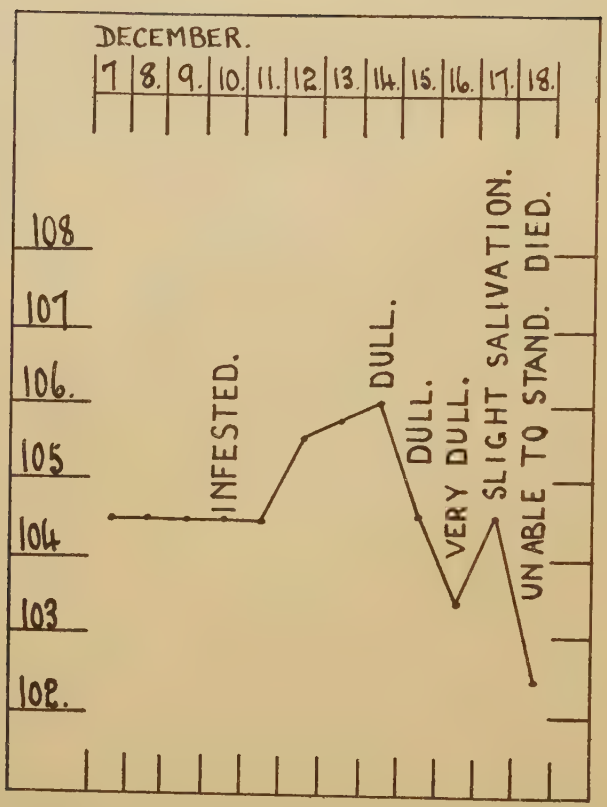
Sheep 277.—This sheep was infested with tick-borne fever infected ticks, and, during the subsequent reaction, was infected with louping-ill infected ticks. The arrows indicate the days on which blood was tested for the presence of louping-ill virus. "O" means "Mouse survived"; "+ T.L.I." means "Died of typical louping-ill."

FIG 4.



Sheep 248.—Infested simultaneously with louping-ill infected and tick-borne fever infected ticks. The sheep developed symptoms of louping-ill and was destroyed when moribund.

FIG 5



Sheep 247.—Temperature reaction and death as a result of infestation with louping-ill infected ticks, *i.e.*, without the presence of tick-borne fever.

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